INHIBITION OF THE BILIARY SECRETION OF METHOTREXATE AS AN APPROACH TOWARD MINIMIZING ITS TOXICITY

by

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This is dedicated to my wife

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The scholar for whom all facts are of significance, and all of equal significance, never understands anything, but nature is kind, and conceals from him that there is anything to understand.

Anonymous

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ABSTRACT

The accumulation of methotrexate in the intestinal lumen was hypothesized to be a major factor contributing to the severity of the gastrointestinal toxicity of this drug. Since the accumulation of methotrexate in the intestinal lumen is due to biliary secretion, the biliary transport of methotrexate was investigated as well as the feasibility of inhibiting this process.

Probenecid and sulfinpyrazone were administered to a group of rats concurrently with methotrexate. Probenecid was found to significantly increase the toxicity of methotrexate, and it was subsequently shown that the major effect of probenecid was to inhibit the biliary secretion of methotrexate.

The kinetics of the biliary transport of methotrexate were then studied under steady state conditions in unanesthesized rats. The maximal rate of biliary transport of methotrexate was found to be about 12 mg/hr. The transport constant, equal to the concentration of methotrexate in the blood which corresponds to a biliary transport rate of one half of the maximal rate, was found to be approximately 3.3×10^{-2} mg/ml.

The inhibition of the biliary transport of methotrexate was also studied under steady state conditions in unanesthesized rats. A value for the inhibition constant, which is equal to the dissociation constant of the probenecid-transport carrier complex,was found to be 2.27×10^{-2} mg/ml.

Inhibiting the major route (biliary transport) of elimination of methotrexate from the blood produced prolonged and elevated blood levels

of the drug. The potentially toxic consequences of prolonged levels necessitated an adjustment in the dose of methotrexate when it was administered concurrently with probenecid. In postulating a modified dose, it was necessary to consider the effect of probenecid on both the renal and the biliary secretion of methotrexate. A graphical relationship between the plasma concentration of probenecid and the sum of renal and biliary clearance of methotrexate was prepared. Using this relationship, a prediction of the area under a methotrexate blood concentration-time curve for an intravenous dose could be predicted as a function of the probenecid plasma concentration.

It was predicted that the area under the blood concentration-time curve of methotrexate following a 5.3 mg/kg intravenous dose, when administered to a rat with a probenecid plasma level of 230 micrograms/ml, would be the same as the area under the blood concentration-time curve for a 25 mg/kg intravenous dose administered to a nonprobenecid treated rat. A dose of 5.3 mg/kg of methotrexate was administered intravenously to a group of five rats concurrently with a constant infusion of probenecid. The area under the blood concentration-time curves were determined, and compared with the predicted area. The average of the measured areas was 16% below the predicted value. The range of the measured areas was from 22% greater to 43% below the predicted value.

The criteria used for evaluating the effect of probenecid on the disposition of methotrexate took into account only pharmacokinetic considerations, and it is important to stress that the studies conducted here do not justify any conclusions as to the therapeutic advantage of the proposed modified dosing procedure over the currently accepted methods for administering methotrexate. In further evaluating this interaction, it will be necessary to conduct studies on leukemic rats and compare the therapeutic and toxic responses of the proposed modified dosing procedure with a currently accepted one.

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INTRODUCTION

Methotrexate is an antineoplastic agent which is used primarily for the maintenance of remission of acute childhood leukemias and for the treatment of choriocarcinoma of the uterus. Though the pharmacological effectiveness of methotrexate has been known for over twenty years, the therapeutic usefulness of this drug was not fully appreciated until more recently when appropriate dosage regimens were developed which enabled a greater therapeutic response to be obtained without exceeding the "maximum acceptable level of toxicity." Like almost all anticancer drugs, methotrexate is a nonspecific agent which is just as toxic to normal as it is to malignant cells. This "maximum acceptable level of toxicity", therefore, is a major consideration which limits how vigorously chemotherapy can be pursued in treating human malignancies. Even though methotrexate is almost accepted as a cure for choriocarcinomas, and its use in maintaining remissions of acute childhood leukemias is proving to be very rewarding, the degree of toxicity which must be accepted by the patient is still substantial.

The toxicity of methotrexate, resulting from its nonspecific arrest of cell replication, occurs primarily in those tissues with rapid cell turnover rates; the most seriously affected tissues are the bone marrow and the mucosa of the gastrointestinal tract. The gastrointestinal disturbances which methotrexate causes, not only result in a great deal of discomfort to the patient, but can progress from hemorrhagic enteritis to the point of intestinal perforation which can lead to a fatal outcome.

Though any drug which arrests cell replication would be expected to be toxic to a tissue with a rapid rate of cell turnover, such as the mucosa of the intestinal tract, in the case of methotrexate an added problem exists; methotrexate is rapidly secreted via the bile into the intestinal lumen, and due to slow absorption, it accumulates at this site. As a result, this susceptible tissue is exposed to much higher concentrations of the drug and for longer periods of time than any other tissue in the body. It, therefore, appears that this distribution phenomenon is a major contributing factor to the severity of methotrexate's intestinal toxicity.

It is hypothesized that if the biliary secretion of methotrexate could be selectively inhibited, this accumulation in the intestinal lumen could be prevented, and the intestinal toxicity minimized.

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LITERATURE COMMENTARY

Structural Characteristics

Methotrexate (4-amino-N^{10}-methylpteroylglutamic acid) is a folic acid antagonist which was originally synthesized along with other analogs of folic acid in an effort to develop a drug effective in the treatment of acute childhood leukemia (1,2). Methotrexate, like folic acid is structurally composed of three basic units: a pteridine ring, para-aminobenzoic acid, and glutamic acid. The essential structural feature which distinguishes methotrexate, as well as the other folate antagonists, from folic acid is the substitution of an amino for a hydroxyl group at the four position of the pteridine ring (Fig. 1). It is of interest to note that the folate antagonist aminopterin is equally as effective as methotrexate as a folate antagonist *in vitro*, but the presence of a methyl group on the 10-nitrogen makes methotrexate a more effective compound *in vivo* (3,4).

Folic Acid Biochemistry

In order to appreciate the biochemical role of methotrexate as a folate antagonist, it is necessary to understand the pertinent features of the biochemistry of folic acid. Only a brief outline of the major biochemical pathways for which folic acid is essential will be presented as numerous reviews of this subject are available (4,5,6,7,8).

Although folic acid is usually referred to as a vitamin, it is not itself involved in any essential biochemical pathways, and is basically a pro-compound which must first be enzymatically converted to an active

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FIGURE 1. Structures of folic acid and the two folate antagonists, methotrexate and aminopterin.

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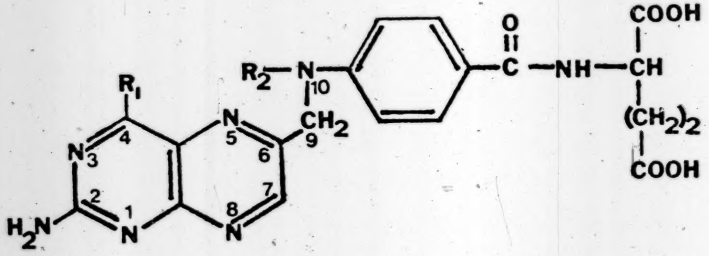

	R_1	R_2
FOLIC ACID	$-OH$	$-H$
METHOTREXATE	$-NH_2$	$-CH_3$
AMINOPTERIN	$-NH_2$	$-H$

form. The first step in this conversion of folic acid is a reduction of the 5,6 double bond of the pteridine ring, yielding dihydrofolic acid. This reaction is mediated by the enzyme dihydrofolate reductase. Though the existence of dihydrofolate has been proven by its isolation, it is very short lived because of its high reactivity, and rapidly undergoes further reduction to tetrahydrofolic acid. This second reduction involves the 7,8 bond of the pteridine ring and is also mediated by the enzyme dihydrofolate reductase (Fig. 2). This biologically important product, tetrahydrofolate, functions as an acceptor and donor of single carbon units in several essential biochemical pathways.

There are three major biochemical pathways for which tetrahydrofolic acid plays an essential role as an acceptor of single carbon units. These include: (1) the biosynthesis of glycine from serine, (2) the decarboxylation of glycine, and (3) the breakdown of histidine to glutamic acid. Two of these pathways, the formation of glycine from serine, and the decarboxylation of glycine to carbon dioxide and ammonia, yield the tetrahydrofolic acid coenzyme methylenetetrahydrofolate. The final step in the breakdown of histidine to glutamic acid involves the removal of the formimino group from formiminoglutamic acid to yield glutamic acid and 5-formiminotetrahydrofolate. The 5-formiminotetrahydrofolate then looses ammonia in the presence of cyclodeaminase to yield 5,10-methyltetrahydrofolate. These three pathways are the major sources of generation of the tetrahydrofolate coenzymes. In addition to these, however, tetrahydrofolic acid can also receive a single carbon unit from formaldehyde or formic acid, but these are relatively unimportant processes. The formation of tetrahydrofolate coenzymes from these five sources is diagrammed in Fig. 3. The different coenzyme forms, with the exception of N^{10}-formyltetra-

FIGURE 2. Reduction of folic acid to tetrahydrofolic acid. This is a two step reduction; dihydrofolate is the intermediate. The first step involves the reduction of the 5,6 bond, and the second reduction involves the 7,8 bond.

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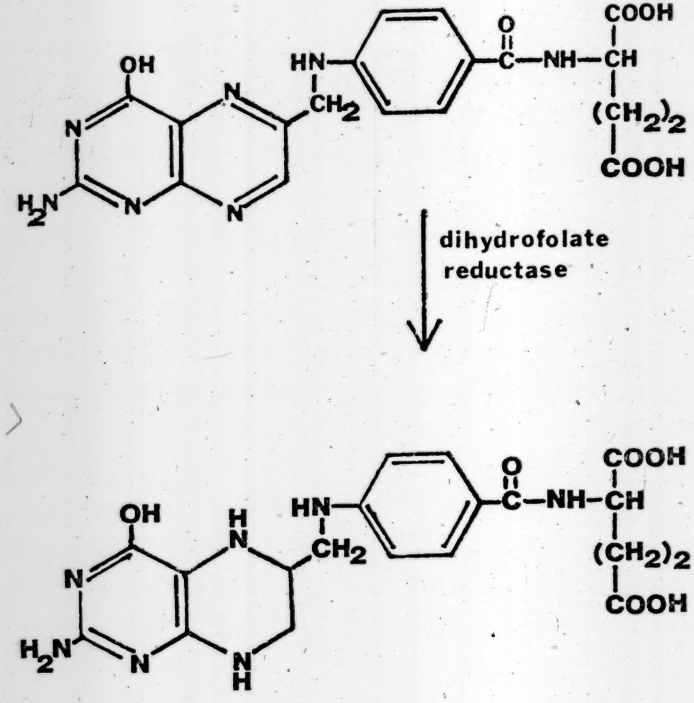

Folic Acid

Tetrahydrofolic Acid

FIGURE 3. Interconversion of tetrahydrofolate coenzymes in relation to the generation and utilization of single carbon units. The reactions shown in the upper half of the diagram yield the various tetrahydrofolate coenzymes, and those reactions shown in the lower half involve the transfer of single carbon units from the coenzyme to the product shown. The structures of the tetrahydrofolate coenzymes are shown in Fig. 4.

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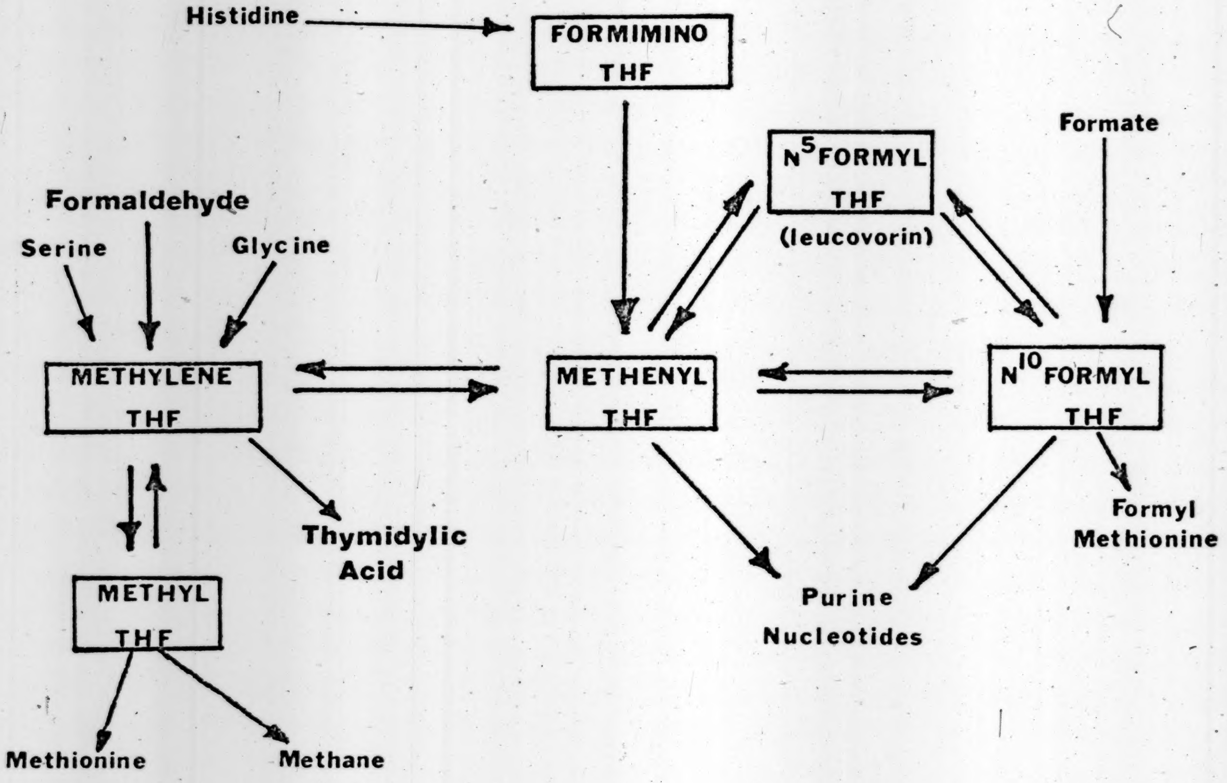
Histidine
FORMIMINO THF
Formate
N^5 FORMYL THF
(leucovorin)
Formaldehyde
Serine
Glycine
METHYLENE THF
METHENYL THF
N^10 FORMYL THF
Thymidylic Acid
Formyl Methionine
METHYL THF
Purine Nucleotides
Methionine
Methane

FIGURE 4. Structures and interconversion of the tetrahydrofolate (THF) coenzymes.

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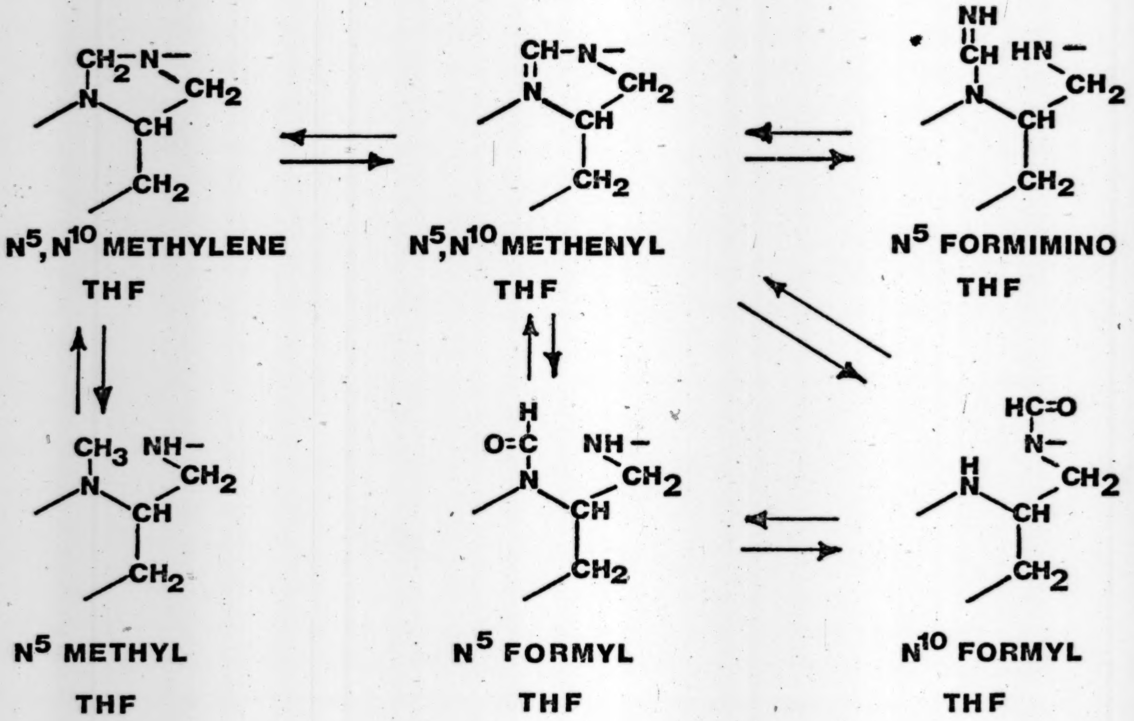
N5,N10 METHYLENE THF
N5,N10 METHENYL THF
N5 FORMIMINO THF
N5 METHYL THF
N5 FORMYL THF
N10 FORMYL THF

hydrofolate are stable and can be isolated. All the different coenzyme forms can be interconverted. These coenzyme forms of folic acid are short lived as they are rapidly utilized as donors of single carbon units in several biosynthetic pathways. The two most important pathways are the *de novo* synthesis of the purine nucleotides, adenylic and guanylic acid, and the methylation of deoxyuridine monophosphate (dUmp) to yield the pyrimidine deoxyribonucleotide, thymidylic acid (Fig. 3).

The purine nucleotides have a common precursor, inosinic acid, and it is in the *de novo* synthesis of inosinic acid that folate coenzymes serve an essential role. Carbons number two and eight of the purine ring are derived from N^{10}-formyl- and methenyltetrahydrofolate, respectively. At an early point in the synthesis of inosinic acid, methenyltetrahydrofolate transfers a formyl group to glycinamide ribonucleotide to supply carbon number eight in the purine ring. At a later step in this synthesis, N^{10}-formyltetrahydrofolate transfers a formyl group to 5-aminoimidazole-4-carboxamide ribonucleotide. This carbon occupies the two position in the purine ring.

The methylation of deoxyuridine monophosphate to form thymidylic acid is another important reaction involving a tetrahydrofolate coenzyme, and this reaction has special significance when considering the mechanism of action of methotrexate. Most reactions which utilize either tetrahydrofolate or tetrahydrofolate coenzymes as donors or acceptors of carbon units result in transfer of a single carbon unit, only. The tetrahydrofolate nucleus is usually unaltered and can be further utilized. In the formation of thymidylic acid from deoxyuridine monophosphate, however, methylene tetrahydrofolic acid functions both as a methyl group donor and as a reducing agent. The resulting dihydrofolic acid is inactive and cannot be

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further utilized until it is again reduced to the tetrahydro form.

Of the other pathways in which tetrahydrofolate coenzymes are involved as carbon donors, the formylation of methionine is the only one of major importance. In this reaction, N^{10}-formyltetrahydrofolate is involved in the transfer of a formyl group to methionyl-tRNA. This formylmethionyl-tRNA has been shown to be essential in the formation of an "initiation complex". This complex is necessary for the initiation of most polypeptide syntheses in bacterial cells. The significance of this reaction in mammalian cells has yet to be established.

Mechanism of Action of Methotrexate

All biochemical pathways involving tetrahydrofolate coenzymes are inhibited by folic acid antagonists. The mechanism of action of folate antagonists was elucidated through very extensive investigations by several groups of different workers. Nichole (9) was the earliest investigator to speculate that methotrexate and the other four-amino analogs of folic acid exerted their effect by preventing the conversion of folic acid to an active substance referred to as Citrovorum factor*. Later studies (10) clearly demonstrated that these 4-amino analogs prevented the reduction of folic acid to the tetrahydro form. Werkheiser (3), using rat liver preparations, found that the inhibition of the enzyme dihydrofolate reductase was proportional to the amount of methotrexate present, and that the enzyme and methotrexate could be stoichiometrically titrated against each other. He clearly demonstrated that by competitively binding the dihydrofolate reductase, methotrexate prevented the reduction of folic acid to the biologically active tetrahydro form. Werkheiser reported a dissociation constant of 3×10^{-11} M for the methotrexate-enzyme complex, suggesting the drug is one of the most tightly bound reversible enzyme inhibitors known.

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* Citrovorum factor was the name given to a factor known to be essential for the growth of Leuconostoc citrovorum 8081 cultures. This was later found to be N^5-formyltetrahydrofolic acid.

The events which occur following the administration of methotrexate may be summarized as follows:

(1) The drug molecules which get into the cells bind tenaciously to dihydrofolate reductase, thus preventing the reduction of folic acid to tetrahydrofolate.

(2) Without the ability to reduce folic acid or dihydrofolate to the tetrahydro form, the levels of tetrahydrofolate coenzymes decrease as the existing coenzymes are used up, and

(3) As a result of this deficiency of tetrahydrofolate coenzymes, DNA synthesis is arrested.

(4) Consequently, cells in the DNA biosynthetic "S" phase of the cell cycle die, and all cells subsequently entering "S" phase die.

Cells which are not undergoing DNA synthesis are not affected by methotrexate. These mature cells are able to synthesize new dihydrofolate reductase, and with replacement of this enzyme, cell replication can again take place and tissue integrity can be maintained. If, however, levels of free methotrexate are maintained, newly synthesized enzyme, as it is formed, will become bound by the drug, and the recovery of susceptible tissues will be prevented (11).

Methotrexate Pharmacokinetics

Until recently, attempts to elucidate the physiological disposition of methotrexate were hampered by the unavailability of a sufficiently sensitive and accurate assay procedure. With the recent availability of highly purified tritiated drug, the distribution and elimination characteristics of methotrexate have been elucidated (12,13,14,15). Bischoff et al. (15) have recently investigated the disposition of tritiated methotrexate in mice, rats, and man. Following either an intravenous or intraperitoneal injection, plasma and tissue levels of the drug were determined at specified times for up to 4 hours after administration. From their data they derived a general mathematical model which describes the distribution and elimination of methotrexate in these species during this time interval. It was found that following parenteral administration, methotrexate is rapidly eliminated from the blood, and becomes greatly concentrated in the intestinal lumen (Fig. 5). Following an intraperitoneal injection of 6 mg/kg of methotrexate to rats, the level of drug in the intestinal lumen rises during the first 2 hours and then slowly declines. The concentration of methotrexate in the intestine, 4 hours after administration, is approximately fifty times that in the plasma. This accumulation of methotrexate in the intestinal lumen is a result of biliary secretion of the drug. Whether or not the methotrexate in the intestinal lumen is significantly reabsorbed or excreted in the feces is an unresolved point. It is, however, apparent that methotrexate is not rapidly removed from the intestinal lumen, and the drug accumulates at this site. As a result, the mucosal cells are exposed to high levels of methotrexate for a much longer period of time than other tissues in the body which are exposed to the drug solely via the systemic circulation.

FIGURE 5. Plasma and intestinal lumen concentrations of methotrexate following a 6 mg/kg intraperitoneal injection in rats. Concentrations are expressed as micrograms of drug per gram of tissue. Data taken from Bischoff _et al_. (15).

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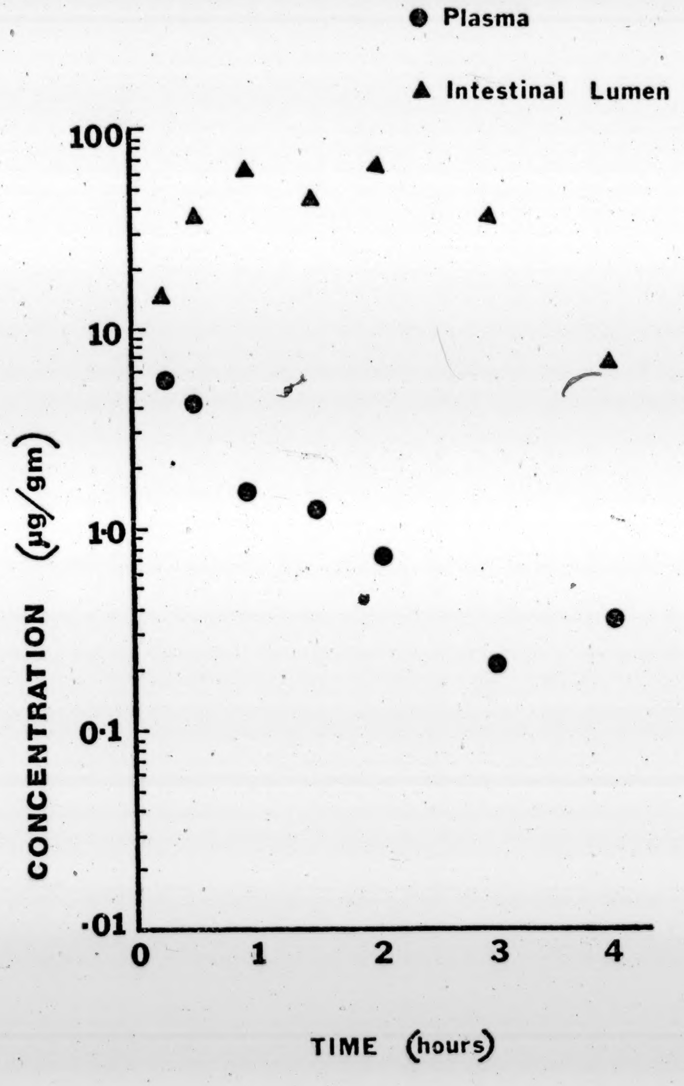
● Plasma
▲ Intestinal Lumen
100
10
1·0
0·1
·01
CONCENTRATION (µg/gm)
0
1
2
3
4
TIME (hours)

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RESEARCH OBJECTIVES

The severity of the gastrointestinal toxicity following methotrexate administration has been recognized for several years. It has been pointed out that frequent dosing of methotrexate can cause hemorrhagic enteritis and even death from intestinal perforation (16). Other nonmalignant tissues such as the bone marrow are also adversely affected by methotrexate, but the problem of gastrointestinal toxicity is the major limitation to the use of this drug in treating malignant diseases (17).

Efforts to minimize the toxic complications of methotrexate administration have primarily been limited to empirical attempts to improve the administration regimen of this drug. In an effort to obtain the maximum therapeutic benefit with a minimum of toxicity, a rather infrequent dosing regimen has been recommended for the use of methotrexate in acute leukemia. It has been found that administration every fourth day allows time for evaluation of the toxicity from the previous dose before subsequent administration (18).

Another approach to this problem would be through the alteration of the physiological disposition* of methotrexate. If the accumulation of methotrexate in the intestinal lumen is prevented, the severity of the gastrointestinal toxicity should be minimized.

An approach to preventing this accumulation would be to inhibit the

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* The term "disposition", as used here, includes all processes involved in a drug's distribution and elimination. Thus all processes after absorption are disposition phenomenon.

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biliary secretion of methotrexate. Though very few reports have appeared in the literature concerning the inhibition of biliary secretion of drugs, it has been reported that compounds which have been used to inhibit renal secretion may have a similar effect on biliary secretion (19).

This study has three primary objectives. The first objective is to find a compound which inhibits the biliary secretion of methotrexate without exerting any other major pharmacological effects. The next step is to characterize the transport process involved, and to relate the degree of inhibition of this process to the concentration of the inhibitor in the blood. Though inhibiting the biliary secretion of methotrexate would prevent accumulation of drug in the intestinal lumen, the problem of toxicity would still be far from solved. By inhibiting this major route of elimination of methotrexate from the blood, prolonged and elevated blood levels would result, and the gastrointestinal mucosa as well as other susceptible tissues would be exposed to toxic levels of drug from the systemic circulation. It would, therefore, be essential to determine what adjustment in dosage would be necessary, since a much smaller dose would elicit the desired pharmacological response if the elimination of the drug from the blood by the biliary route were inhibited. The third objective, therefore, is to determine what would be a therapeutically equivalent dose. In considering the adjustment in dosage, it would be necessary to take into account any other effects which the inhibitor of biliary transport had on the total body clearance of methotrexate.

ANALYTICAL METHODS

Prior to conducting studies on the biliary secretion of methotrexate and its inhibition, it was necessary to be able to measure the concentration of methotrexate in the blood and bile samples. It was also necessary to be able to measure the plasma concentration of the compound used as an inhibitor of the biliary secretion of methotrexate. In this section are presented the analytical techniques utilized to measure the concentration of methotrexate in blood, bile and urine samples, and for measuring the plasma concentration of probenecid (the compound used to inhibit the biliary secretion of methotrexate).

Probenecid Assay

Materials:

A.) Reagents - 1,2-dichloroethane A.C.S. reagent grade obtained from Matheson, Coleman and Bell; 2.5N NaOH; 3.0N HCl; citrate-phosphate buffer, pH 4.8, prepared from solutions of 0.1 M citrate and 0.2 M disodium phosphate.

B.) Drug - Probenecid powder obtained from Merck Sharp & Dohme Research Laboratories.

C.) Equipment and Instruments - International Bottle Shaker; Beckman DU Spectrophotometer; International Centrifuge.

Procedures:

A procedure similar to that of Dayton and Yu (20) was utilized for the assay of probenecid in rat plasma samples. At appropriate times, one to one and a half ml of blood was drawn into a heparinized syringe from

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the rat under study. The blood sample was centrifuged to separate the plasma from the cells, and a half ml of plasma was frozen. It was stored in a freezer until it was convenient to proceed with the assay. This was usually a period of less than three days.

After allowing time for the frozen plasma sample to thaw, 3 ml of distilled water and 1.5 ml of 3.0N HCl were added. Twenty five ml of 1,2-dichloroethane was next added and the tube shaken at a rate of 220 cycles per minute for one hour. Following shaking, the sample was centrifuged to separate the organic and the aqueous layers, and 23 ml of the 1,2-dichloroethane layer was removed to another centrifuge tube. To this 23 ml aliquot was added 25 ml of citrate-phosphate buffer (pH 4.8). This tube was shaken for another half of an hour and centrifuged to separate the layers. From this tube was removed 20 ml of the 1,2-dichloroethane layer, which was transferred to a tube containing 5 ml of 2.5N NaOH. This tube was shaken for half of an hour and then centrifuged. The absorbance of the NaOH phase was determined at 242.5 nm on a Beckman DU spectrophotometer. In order to compensate for the components in the plasma which might interfere with the assay procedure, by absorbing at 242.5 nm, a control was used. The control was an 0.5 ml plasma sample obtained from the specific rat under study, before probenecid was administered. The absorbances of the plasma samples were corrected by substracting the absorbance of the control. The concentration of probenecid in each plasma sample was then determined from a standard curve (Fig. 6), prepared using probenecid samples of known concentrations. These had been carried through the same extraction procedure.

FIGURE 6. Standard Curve for probenecid in 2.5N NaOH. Absorbance was measured at 242.5nm.

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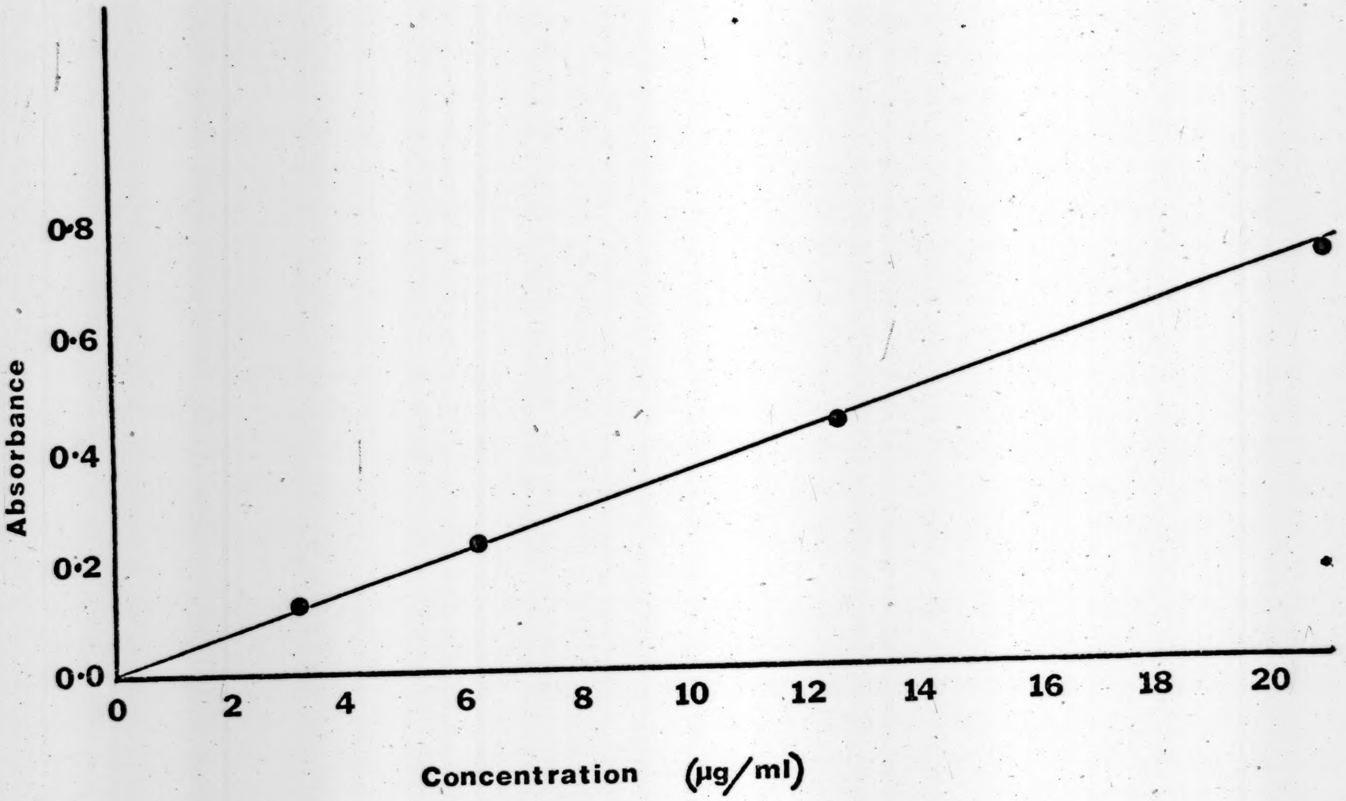
Absorbance
0·0
0·2
0·4
0·6
0·8
0
2
4
6
8
10
12
14
16
18
20
Concentration (μg/ml)

Methotrexate Assay

Materials:

A.) Methotrexate - Nonradioactive methotrexate was obtained as a powder from Nutritional Biochemical Corporation; Radioactive methotrexate -3',5',9-$^3H_3$ sodium salt was obtained from Amersham/Searle Corporation. The labeled compound had a specific activity greater than 3 Ci/mmole, and a guaranteed purity of 98%.

B.) Scintillation Cocktail - Insta-Gel Emulsifier obtained from Packard Instrument Company.

C.) Water-^3H Standard - Tritiated water with a specific activity of 10.4 microCi/gram was obtained from Amersham/Searle.

D.) Equipment and Instruments - Packard Tri-Carb Model 3375 Liquid Scintillation Counter; Packard Tri-Carb Model 305 Sample Oxidizer; Parr Instrument Corporation Model 2811 Pellet Press.

Procedures:

A.) Sample Preparation

To facilitate treatment of blood samples, small sacks were prepared from 9 cm lengths of ¼ inch diameter cellulose dialysis tubing. Following wetting, one end of these sections of dialysis tubing was closed securely by tying a knot. These casings were then dried and weighed. Following removal of a blood sample from a rat which had been injected with tritiated methotrexate, the blood was transferred to a casing. The filled casing was then weighed and the weight of the blood was determined by subtracting the weight of the empty dialysis casing. The volumes of the blood samples were determined by dividing the weight of blood by 1.05, which was assumed to be the average specific gravity of the rat blood (21).

Bile and urine samples were also collected in sacks prepared from dialysis tubing. Regenerated cellulose dialysis tubing with a cross section of 3/4 inches was cut into 10 cm sections and sacks were prepared from them in the same manner as the blood collection sacs. Bile and urine were collected in separate sacks during specified time intervals. The volume of bile and urine samples were not measured. The samples were allowed to dry by setting under a hood for at least 24 hours. The blood samples were also dried in the same manner. The dialysis casing sacks containing dried samples of bile, urine, and blood were then wrapped in Whatman No. 1 filter paper and pressed into pellets of about 3/8 inch in diameter. These pellets were combusted on a Packard Sample Oxidizer using an instrument setting for tritium samples. Following combustion, the Sample Oxidizer automatically collects the tritium label as tritiated water and allows it to flow into a scintillation vial. Scintillation cocktail is then automatically added to the vial. The scintillation vial containing the tritium label from the combusted sample and scintillation cocktail were then removed from the oxidizer, shaken, and refrigerated for 24 hours.

In order to determine the efficiency of recovery of radiolabel from the oxidation procedure, a control study was conducted. Using a 5 microliter disposable pipet, 10 microliters of a tritiated water standard was placed in a counting vial containing 10 ml of scintillation cocktail. Using the same 5 microliter pipet, 10 microliters of the tritiated water was placed on a filter paper pellet. This was done in duplicate. The pellets were oxidized and counted. The recovery was determined by comparing the disintegrations per minute observed from the oxidized pellets with the disintegrations per minute in the 10 microliters which were added directly

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to the counting vial. It was found that recovery from the oxidation procedure was essentially 100%.

B.) Counting Methods

The initial step which must be taken when using liquid scintillation counting techniques is to provide a means for correcting for variable quenching of samples. Quenching is due to impurities in the sample which interfere with the scintillation process. The volume independent automatic external standardization feature, which was available on the scintillation counter used, was utilized for all quench corrections. The external standard is a compound source of americium-241 and radium-226. This is pneumatically transported from a shielded storage chamber into the counting chamber which contains a sample vial. The standard is then counted. If there is no quenching due to the scintillation vial or contaminants in the sample, the counting rate as observed in two preset counting windows will be the same. If there is quenching, however, there will be a downward shift of the pulse height spectrum toward the low energy region, and the counting rate in the window with the lower setting will be greater. A ratio of the counting rate observed by the two windows is then recorded. This ratio of the upper to the lower window, which is always less than one when there is quenching, is referred to as the Automatic External Standard (AES) Ratio. This ratio is linearly related to counting efficiency; the lower the counting efficiency, the smaller is the ratio (22).

A quench correction curve was prepared as follows: to each of 7 scintillation vials was added a volume of 10 ml of Insta-Gel emulsifier, followed by the addition of 0.1 ml of a tritiated water standard. Each vial was then counted, and a correction for pipetting error was determined by counting each vial. Using water as a quenching agent, different volumes were added to each vial. Each vial was then again counted and the counting rate and the AES ratio were recorded. The preset tritium counting window was used for all determinations. These data are presented in Table I.

A quench correction curve was prepared by plotting the automatic external standard ratio against the counting efficiency for each vial (Fig. 7). The counting efficiency was determined by dividing the measured counts per minute in each vial by the known disintegrations per minute which were added originally. This curve was reconstructed from time to time as the relationship between the ratio and the counting efficiency tended to change over long periods of time. This was due both to readjustments in the normalization component of the machine and to unpredictable machine drift.

All scintillation vials were counted either for a preset time of 10 minutes or for a preset count of 900,000 counts. Besides the sample counting rate, the external standard ratio was recorded for each vial. The vials were counted once during each of 2 cycles. From the previously prepared quench correction curve, the efficiency corresponding to the average external standard ratio was determined, and the actual disintegrations per minute were calculated for each vial.

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TABLE I

DATA FOR PREPARATION OF QUENCH CORRECTION CURVE

Vial Number	CPM[a]	Pipetting Error	DPM[c]	Water Added	CPM With Water	% Efficiency	AES[d] Ratio
1	$2.04X10^5$	+1.6%	$5.59X10^5$	0.0 ml	$2.33X10^5$	41.8%	.7131
2	$2.02X10^5$	+0.5%	$5.53X10^5$	0.6 ml	$2.00X10^5$	36.2%	.6449
3	$1.99X10^5$	-1.2%	$5.43X10^5$	0.8 ml	$1.92X10^5$	35.3%	.6305
4	$2.04X10^5$	+1.3%	$5.57X10^5$	1.0 ml	$1.95X10^5$	35.0%	.6290
5	$2.02X10^5$	+0.3%	$5.52X10^5$	1.2 ml	$1.89X10^5$	34.3%	.6121
6	$2.01X10^5$	-0.2%	$5.49X10^5$	1.6 ml	$1.79X10^5$	32.5%	.5943
7	$2.04X10^5$	+1.5%	$5.58X10^5$	2.0 ml	$1.74X10^5$	31.2%	.5776

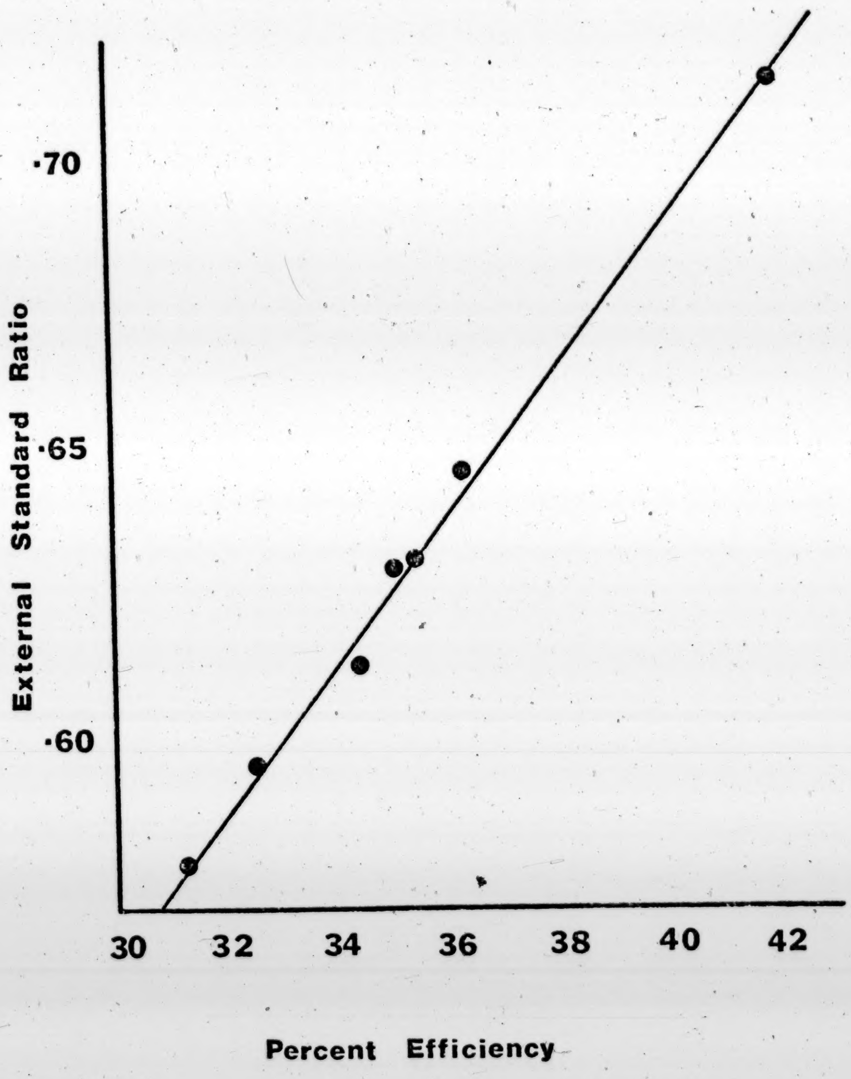
[a] Counts per minute before adding water as quencher

[b] Pipetting error is the percent deviation of each vial from the average counts per minute for all the vials, since it was attempted to pipet the same amount into each vial.

[c] To each vial was added $5.50X10^5$ DPM. The values given above are corrected for pipetting error.

[d] Automatic External Standardization Ratio

FIGURE 7. Quench curve for calculating efficiency of counting from external standard ratio.


·70
·65
·60
External Standard Ratio
30
32
34
36
38
40
42
Percent Efficiency

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METHOTREXATE PURIFICATION

Due to the impurity and relative instability of commercially obtainable methotrexate, it was necessary to purify the compound before conducting any studies.

Materials and Methods

Materials:

A.) Chromatographic Columns - Two different columns were used for the purification of methotrexate. A laboratory buret which had an internal diameter of 1.2 cm and a length of 30 cm was used when small quantities of drug were being purified. For larger quantities of drug, a column 48 cm long and with a 3.0 cm internal diameter was used.

B.) Diethylaminoethylcellulose (DEAE) - DEAE was obtained from BioRad Laboratories as Cellex-D^R. The DEAE was of medium capacity with 0.75meq/gm. Before use it was necessary to thoroughly wash the DEAE. The DEAE was washed with 1 N NaOH, distilled water, 1 N HCl, and then again in distilled water. This series of washings was then repeated a second time, and the DEAE was washed repeatedly with 0.1 M ammonium bicarbonate buffer (pH 8.3). Following at least 3 washings with this buffer, the DEAE was lightly packed into a glass column. Following packing, the column was wrapped with aluminum foil to prevent photodecomposition of methotrexate from occurring during the chromatographing procedure.

C.) Fraction Collector - A Golden Retriever model 327 fraction collector with a manometer electrode assembly was obtained from Instrumentation Specialities Company. A 10 ml volumetric siphon was obtained with the machine.

D.) Lyophilizer - A Virtis Company Freeze Mobile model 10-146MR-BA with a vacuum drying chamber model 10-MR-SA was used for all lyophilization procedures.

E.) Ammonium Bicarbonate - Ammonium Bicarbonate, analytical reagent grade, was obtained from Mallinckrodt Chemical Company.

Methods:

Commercially obtained methotrexate was dissolved in a minimum amount of 0.1 M ammonium bicarbonate buffer and transferred to a column. Elution was then carried out using an increasing concentration gradient of pH 8.3 ammonium bicarbonate buffer. The gradient was produced by periodically adding 0.4 M buffer to a well stirred reservoir, which initially was filled with 0.1 M buffer. The column flow rate was approximately 0.5 ml per minute. Using an automatic fraction collector with a 10 ml volumetric siphon, fractions of 10 ml were collected as they eluted from the column. The fraction collector and the eluted fractions were protected from light by a cardboard shield which covered the entire apparatus.

Following completion of the elution of methotrexate from the column, the fractions were analyzed using a Beckman DU spectrophotometer. The absorbance of each fraction at 302 nm was measured in an effort to determine the presence of impurities which were not readily visible and to establish that the separation of methotrexate from these impurities was complete. The location of methotrexate fractions and those of the primary contaminant were readily visible due to their deep yellow color. When purifying radiolabeled drug, following elution from the column, the location of radiolabel was determined by adding 100 microliters of each fraction to a scintillation counting vial containing 10 ml of InstaGel scintillation cocktail. The vials were then counted on the scintillation spectrometer. This was done to establish the completeness of separation of radiolabeled methotrexate from radioactive impurities.

Only for purposes of determining the existence and location of contaminants were the absorbance and the presence of radiolabel determined for each fraction. As a general rule, the tubes containing the methotrexate were visually located and collectively frozen in a beaker. The other fractions were then discarded. The contents of the beaker was then lyophilized and the lyophilate was stored in a dessicator at -20°C. Methotrexate was usually used within 3 weeks of the date of purification.

Results and Discussion

The lack of purity of commerically available methotrexate has been well documented. Tisman and Winsten (23), using electrophoretic methods, found 3 contaminants present in a methotrexate preparation which was marketed for medicinal use. At a pH of 8.3, they found that 2 of these impurities migrated in the same direction as methotrexate, suggesting they were anionic in nature, and that the third one, being unionized, remained at the origin. Gallelli and Yokayama (24) also found 3 major contaminants in a supposedly pure sample of methotrexate U.S.P. Following separation of these contaminants on an anionic exchange column, they determined the ultraviolet absorption spectrum of each at a pH of 8.3. In the spectrum of each species, they reported the presence of 2 absorption maxima, one at 260 nm and the other at 302 nm. The peak at 260 nm was observed in the spectrum of each species. The 302 nm peak, however, was absent in the spectrum of one of the impurities. These 2 absorption maxima were of almost equal intensity in all 3 species in which they were present. One of the impurities was tentatively identified as N^{10}methylpteroylglutamic acid, and it was reported that this contaminant accounted for approximately one half of the total impurities present.

Besides the lack of initial purity of methotrexate, it has also been shown that pteridine containing compounds such as methotrexate are unstable chemically and rapidly decompose under certain conditions. Jukes (25) reported that most compounds containing a pteridine moiety are light sensitive and readily undergo photodecomposition if not adequately protected from exposure to light. In addition to photodecomposition, Jukes also reported that exposure to the air can result in oxidation of the pteridine ring. These two problems, photodecomposition and oxidation, can both occur even when these compounds are stored as lyophilized powders. In solution, an additional problem exists; pteridine compounds are sensitive to pH. Jukes reported that either high or low extremes of pH can lead to various types of transformations.

Thus this problem of decomposition, as well as the lack of initial purity, necessitates the purification of methotrexate prior to any studies on its physiological disposition. A column chromatographic procedure similar to the method reported by Oliverio (26) was utilized.

Prior to purification the tritiated methotrexate was diluted with nonlabeled drug. Since the labeled and the nonlabeled methotrexate were obtained from different sources, it was not expected that they would be of equal purity, nor that they would necessarily contain the same impurities. The purity of the tritium labeled methotrexate was reported by the supplier to be 98%, as determined by paper chromatography using two different solvent systems: 0.5% sodium carbonate solution, and a mixture

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of equal parts of n-butanol, pyridine, and water. It was also reported that approximately 35% of the tritium activity of the labeled methotrexate is present at the 9-methylene group, and the other 65% is on the phenyl ring at the 3',5' positions. The supplier of the nonlabeled drug, however, made no claims as to its purity.

After chromatographing methotrexate, the absorbance at 302 nm, as well as the radioactivity in a 100 microliter aliquot of each fraction were determined. In addition to the impurities which could be separated from methotrexate by chromatographic methods, there was also a substantial amount of material which was insoluble in the pH 8.3 buffer system, and thus remained on top of the column.

Figure 8 illustrates a typical chromatogram of absorbance following separation of methotrexate from its impurities. From the same fractions, a chromatogram of counts per minute in 100 microliter aliquots was also prepared (Fig. 9). With the exception of peaks numbered 2 and 6 on the 2 chromatograms, the peaks with corresponding numbers came off with the same elution volumes. The fractions containing methotrexate (peak 5), as well as those comprising peaks 1 and 4, were yellow in color. The major contaminant observed (peak 6) in the absorbance chromatogram was eluted more slowly than methotrexate. This species corresponds to the impurity which Gallelli and Yokayama tentatively identified as N^{10}methylpetroylglutamic acid. Quantitatively, peaks 1 through 4 on the absorbance chromatogram represented only a very minor fraction of the total species present in the original mixture. The fractions containing methotrexate and those containing the primary contaminant had absorbances which approached infinity, indicating that these species were present in relatively high concentration. The major contaminant observed on the absorbance

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FIGURE 8. Chromatogram of absorbance following purification of methotrexate by column chromatography. The absorbance of each fraction was measured at 302 nm. Peak five represents methotrexate.

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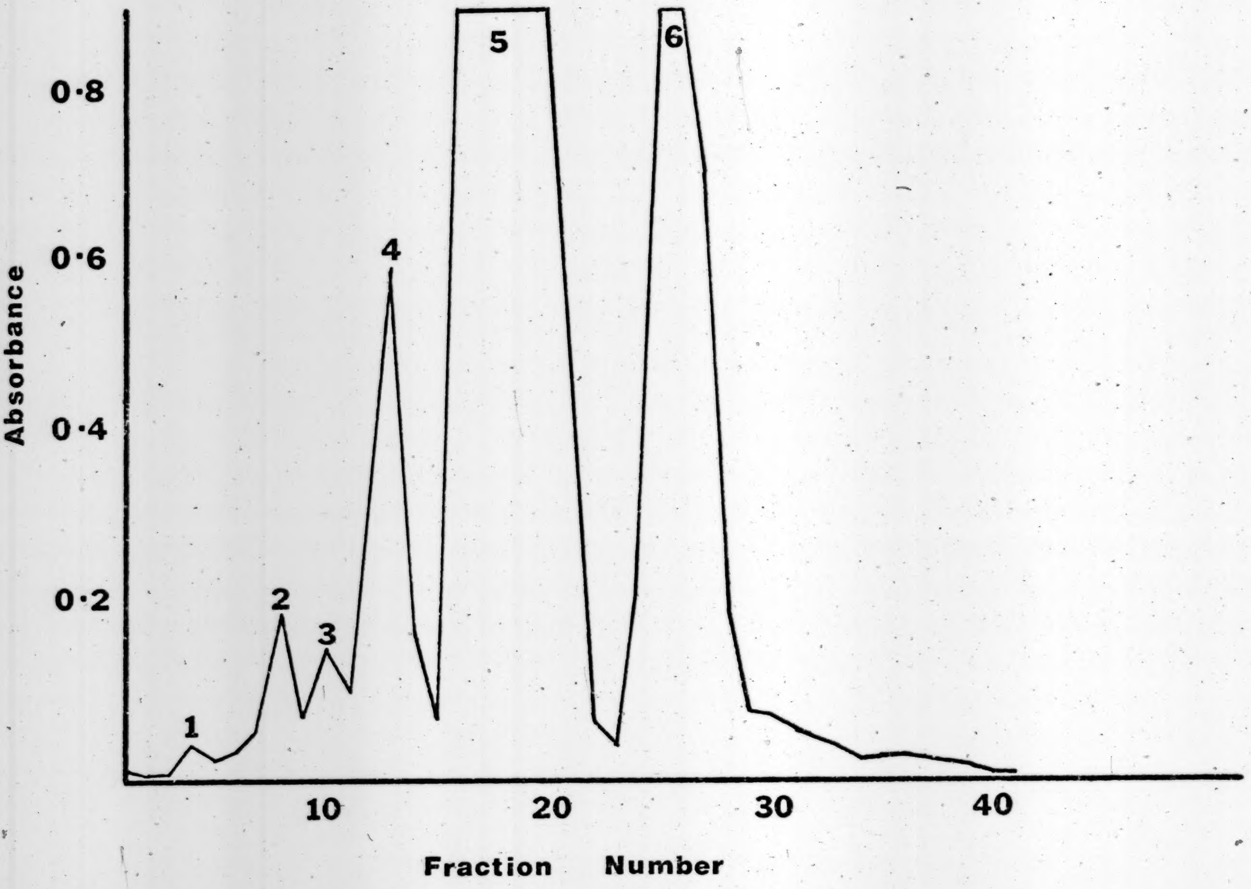

FIGURE 9. Chromatogram of tritium activity following purification of methotrexate by column chromatography. The counts per minute of 100 microliter aliquots of each fraction were measured. Peak five represents methotrexate.

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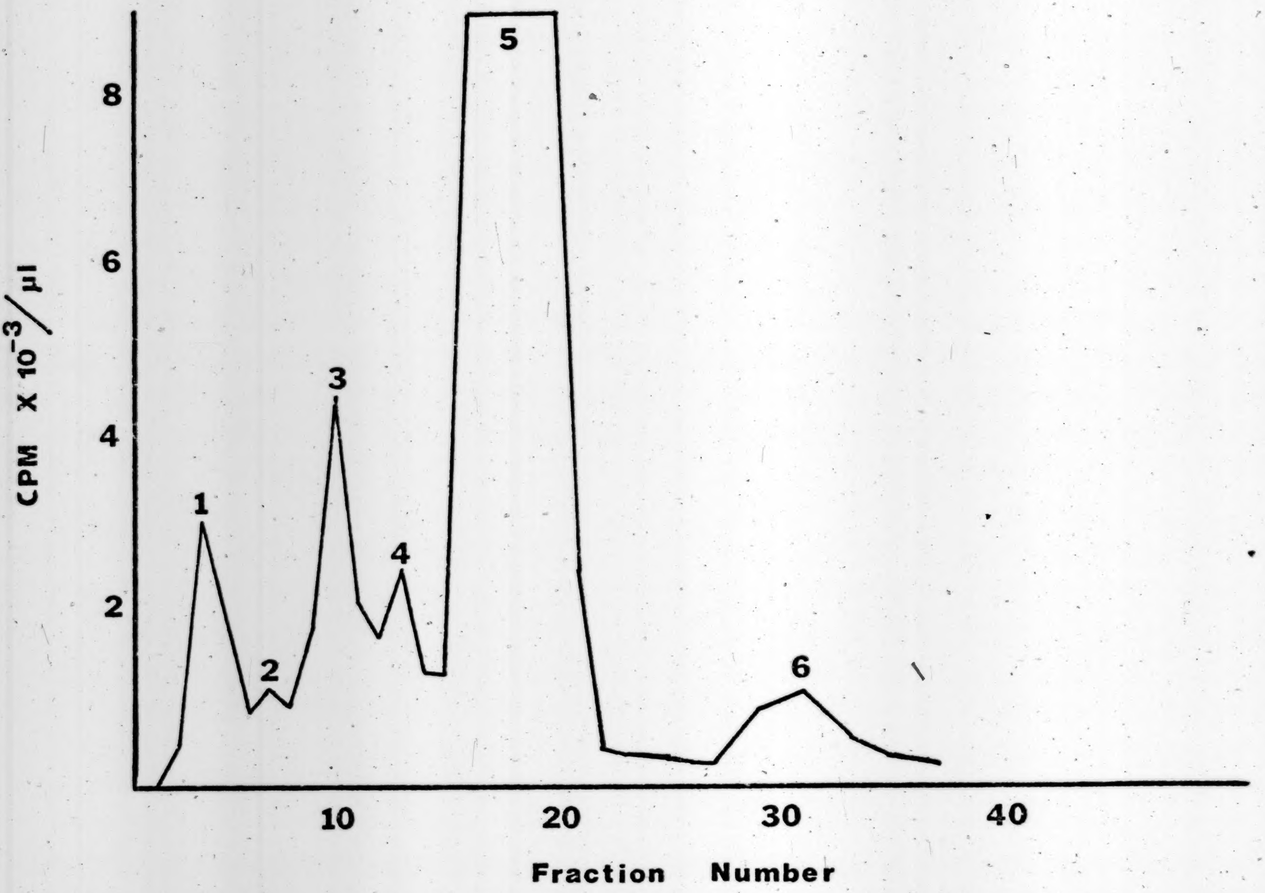

chromatogram was not observed when the presence of tritium label was measured. Since labeled methotrexate is tritiated on the pteridine ring, as well as on the phenyl ring, it is unlikely that an unlabeled decomposition product which would absorb at 302 nm could be produced. This impurity is most likely absent from the relatively well-purified tritium labeled preparation, and only exists as a contaminant of the nonlabeled product. As expected, methotrexate was observed in the same fractions on both chromatograms.

Following separation, the methotrexate containing fractions were collected, frozen, and lyophilized. They were then rechromatographed; only one peak was observed.

EXPERIMENTAL PROCEDURES

The experimental protocal for each set of studies, along with the material necessary, is presented below.

Toxicity Screening Studies

Materials:

A.) Drugs - (1) Sodium methotrexate parenteral solution, marketed by Lederle Laboratories Division of American Cyanamide was used for these studies. The parenteral solution was marketed in two ml vials containing 25 mg/ml of methotrexate. (2) Probenecid was obtained as a powder from Merck Sharp and Dohme Research Laboratories. Probenecid injectable solution was prepared by dissolving the weighed powder in a few drops of 1.0 N NaOH, and then adjusting the pH to 7.4 with a pH 7.0, 0.5M phosphate buffer. This solution was then diluted to the desired volume with normal saline. The final concentration of the probenecid injectable solution was 20 mg/ml. (3) Sulfinpyrazone was obtained as a powder from Geigy Pharmaceuticals. The sulfinpyrazone was dissolved in 1.0 N NaOH, as was the probenecid, adjusted to pH 7.4, and diluted to the desired volume with normal saline. The final injectable solution contained 10 mg/ml.

B.) Rats - Male Sprague Dawley rats, weighing 225 to 275 grams, were used. They were obtained from Simonsen Laboratories of Gilroy, California.

Procedures:

Three groups of ten rats each were injected intraperitoneally with 12.5 mg/kg of methotrexate. One of these groups received, in addition to the methotrexate, an intraperitoneal injection of 200 mg/kg of probenecid.

The probenecid was administered as two separate injections of 100 mg/kg each. One dose of probenecid was administered 20 minutes before the methotrexate was injected, and the other was administered 20 minutes after. Another group of rats was injected with 100 mg/kg of sulfinpyrazone in addition to the methotrexate. This was administered intraperitoneally as a single bolus 20 minutes before the administration of methotrexate. The third group of rats, serving as a control, received only methotrexate.

The rats within each group were paired and placed in cages with one pair per cage. Feces were collected daily and assayed for occult blood by the Benzidine test (27). Observations of toxic manifestations were recorded.

The above procedure was repeated a second time, and the dose of methotrexate was increased to 25 mg/kg. Only two groups of 10 rats were used. One group received only methotrexate, and the other group was administered methotrexate, and probenecid was administered as previously described.

Methotrexate Bolus Studies - Effect of Probenecid

Materials:

A.) Polyethylene Tubing - Polyethylene tubing (P.E. 90) from Clay Adams was used for this study. This tubing had an internal diameter of 0.034 inches and an external diameter of 0.050 inches.

B.) Drugs - (1) Purified tritiated methotrexate was dissolved in a few drops of 0.1 M ammonium bicarbonate buffer (pH 8.3) and dissolved with normal saline for injection. Prior to injection of the tritiated methotrexate, a 100 microliter sample of the injectable solution was transferred to a dialysis casing sack. Following drying, this sample was oxidized and counted in the same manner as the blood samples. Determination of the disintegrations per minute in this 100 microliter sample enabled conversion of the disintegrations per minute in the blood samples to micrograms of drug. (2) Probenecid was prepared in the same manner as described in the procedures for the Toxicity Screening Studies.

C.) Rats - The rats used for this study were male Sprague Dawley weighing between 450 and 525 grams. They were obtained from the same vendor as the rats used in the Toxicity Screening Studies.

D.) Restraining Cage - Plastic rat restraining cages (28) were used.

Procedures:

Since this series of studies required frequent sampling of blood from rats, it was necessary to cannulate a vein, in order to have a dependable blood sampling site. Cannulae were prepared from 30 cm lengths of P.E. 90 tubing. The end which was to be inserted into the vein was beveled, and on the side of the cannula opposite the bevel, a small hole was cut. It had been found that if the beveled opening of the cannula were in close proximity to the wall of the vena cava, the wall would be drawn up against the end of the cannula, thus blocking the opening and preventing blood withdrawal. With an opening on both sides of the cannula, blood could be drawn through either side, and blockage was avoided. Three centimeters from the beveled end a 0.5 cm length of P.E. 160 tubing (inner diameter of 0.045 inches) was placed around the cannula and fastened in place with epoxy resin. This served as a collar to facilitate securing the cannula to the vein. Since the internal diameter of the collar was smaller than the external diameter of the cannula, it was necessary to cut the collar lengthwise in order to enable the cannula to fit

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through. The cannula was then filled with heparinized saline and the end from which blood samples were to be withdrawn was plugged with a piece of wire, 1 cm in length, cut from an ordinary #1 paper clip.

The surgical procedure for the insertion of the cannula through the jugular vein into the vena cava required approximately 15 minutes, including the time necessary for suturing the entry incision.

Initially the rat was anesthesized with 50 mg/kg of sodium pentobarbital (Nembutal^R) which was administered intraperitoneally. The upper chest and neck region as well as the dorsal part of the head, between the ears, were shaved with an Oster Animal Shaver, and washed with isopropanol. The rat was then placed on a surgery board with its legs secured with rubber bands which were fastened to the board with tacks. A median ventral incision was made in the neck about 3 to 4 centimeters in length, and the fat and fascia were teased apart with blunt forceps until the jugular vein was exposed on the right side of the neck. A 1 centimeter segment of the vein, just anterior to where it descended behind the pectoralis major, was exposed and cleaned. A ligature was then tied at the anterior end of the cleaned segment to stop the venous blood flow. A second ligature was placed under the vein posterior to the first one, but was not tied securely until after the cannula had been inserted. Using microdissecting scissors, a small incision was made in the vein between the two ligatures. The cannula was then inserted into the vein until the collar was at the site of the incision. The posterior ligature was tightened around the vein and tied, securing the cannula in place. A third ligature was then tied around the cannulated vein at the site of the collar to prevent the cannula from slipping out.

The integrity of the cannula was tested by withdrawing blood into it;

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the cannula was washed clear of blood by injecting a small volume of heparinized saline into the animal. The exterior end of the cannula was sealed with a piece of wire as previously described.

The rat was then turned over and a small cutaneous incision was made on the dorsal side of the head between the ears. A 14 gauge hypodermic needle was forced through this incision and subcutaneously around the side of the head between the eye and ear until it appeared in the area of the incision on the ventral side of the neck. The cannula was threaded through the needle and out the cutaneous opening between the ears. The needle was then removed, leaving the cannula exteriorized through the skin on the dorsal side of the head. The ventral incision was sutured, and the rat was placed in a restraining cage and allowed to recover from anesthesia.

The procedure followed for this study was the same as that followed for the Toxicity Screening Studies, with two exceptions: the rats were cannulated for blood sampling and the drugs were administered intravenously. Methotrexate was administered in a dose of 25 mg/kg and probenecid was administered as 2 boluses of 100 mg/kg, 20 minutes before and 20 minutes after the methotrexate was administered.

At specified times, blood samples were withdrawn from the rat through the cannula. Prior to taking a blood sample, however, it was essential to remove the heparinized saline from the cannula to prevent dilution of the blood sample. The volume in the cannula was found to be 0.2 ml. Following removal of blood, the cannula was again filled with heparinized saline and replugged.

Throughout the study the rats were allowed water, but food was withheld.

Separation of Methotrexate From Nonmethotrexate Components in Plasma

Materials:

A.) Membrane Filter Cones - Centriflo^R filter cones from Amicon Corporation were used for deproteinating plasma. These cones retain species with molecular weights greater than 50,000.

B.) Chromatography Paper - Chromatographic strips 3 centimeters in width and 46 centimeters in length were cut from Whatman No. 3MM chromatography paper which was obtained in 46 x 57 cm sheets.

Procedures:

Rats, having been cannulated for blood collection as previously described, were injected intravenously with 25 mg/kg of tritiated methotrexate. At specified times 4 ml of blood was drawn into a heparinized syringe via the jugular cannula. Because of the large volume of blood removed, no more than 3 samples were taken from any one rat.

The scheme shown in Fig. 10 was then followed for separation of the labeled components in the plasma. The blood sample was initially centrifuged to separate the plasma from the red blood cells. The plasma was then removed to a Centriflo^R membrane cone, and the red blood cells, which remained in the centrifuge tube, were washed with normal saline. An aliquot of the plasma was removed to a dialysis casing for later assay, in order to determine the concentration of total radioactivity in the plasma at the specified sampling times. Following centrifugation to separate the cells from the saline wash, the saline fraction was added to the plasma water filtrate. A plasma water filtrate was obtained by centrifugation. Water was then added to the residue in the cone, and following agitation, the cone was again centrifuged. The combined filtrates were

FIGURE 10. Scheme for the separation of methotrexate from nonmethotrexate components in plasma.

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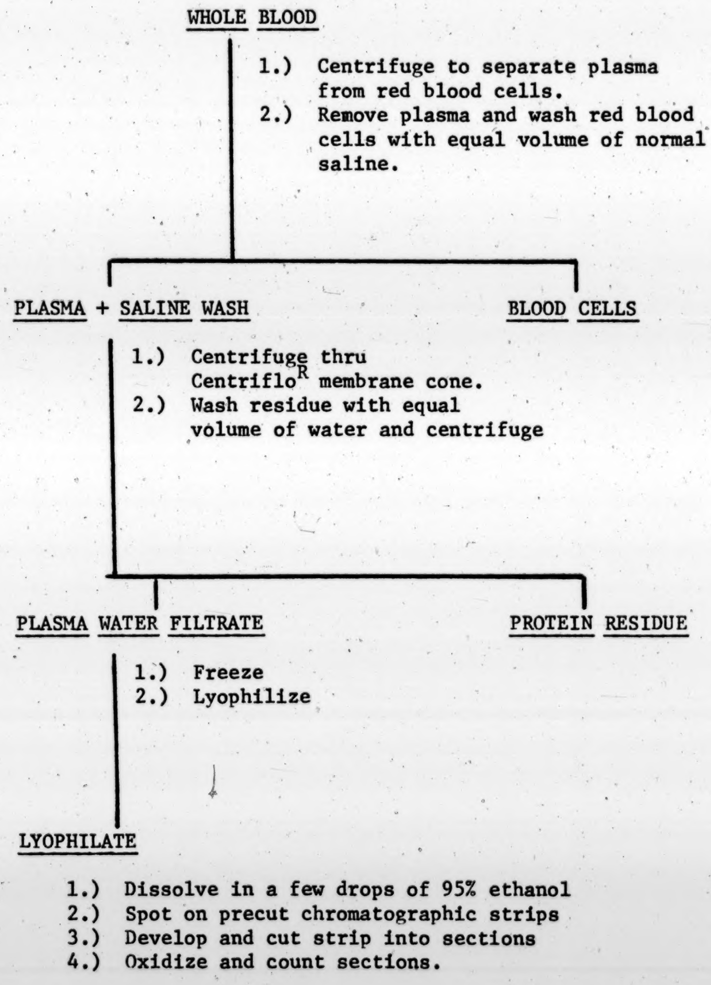

WHOLE BLOOD

1.) Centrifuge to separate plasma from red blood cells.
2.) Remove plasma and wash red blood cells with equal volume of normal saline.

PLASMA + SALINE WASH

BLOOD CELLS

1.) Centrifuge thru Centriflo^R membrane cone.
2.) Wash residue with equal volume of water and centrifuge

PLASMA WATER FILTRATE

PROTEIN RESIDUE

1.) Freeze
2.) Lyophilize

LYOPHILATE

1.) Dissolve in a few drops of 95% ethanol
2.) Spot on precut chromatographic strips
3.) Develop and cut strip into sections
4.) Oxidize and count sections.

frozen and lyophilized.

The lyophilate containing the tritiated methotrexate, other labeled components, and plasma salts was then dissolved in a few drops of 95% ethanol, and the alcoholic solution was spotted on a chromatographic strip. The chromatograms were developed at room temperature by ascending paper chromatography with 0.1 M phosphate buffer (pH 7.0) as solvent in a sealed chamber. The solvent front was allowed to travel to a height of 30 cm. After drying, the strips were cut into sections of 1 cm in length, oxidized, and the amount of radiolabel in each section determined by liquid scintillation counting.

Methotrexate Clearance Study - Effect of Probenecid

Materials:

A.) Arterial Cannula - A 30 cm section of polyethylene tubing (P.E. 20) was used for preparation of the arterial cannula. This tubing had an internal diameter of 0.015 inches and an external diameter of 0.043 inches. The end of the cannula which was inserted into the artery was beveled and a collar was affixed 3 cm from this end. This collar was prepared in the same manner as the collar for the jugular cannula described in a previous section. Bending the beveled end of the cannula to introduce it into the artery has been used successfully elsewhere (29), but proved unsuccessful here, and was not done.

B.) Bile Cannula - A 12 cm section of polyethylene tubing (P.E. 10) was used for preparation of the bile cannula. This tubing had an internal diameter of 0.011 inches and an outer diameter of 0.024 inches. One end was beveled, and 2 cm from this end a collar was fastened. This collar was prepared from an 0.5 cm section of P.E. 90.

C.) Urinary Bladder Cannula - A six cm section from the tubing of a Hyland TYT253 Administration Set was used for preparation of a bladder cannula. One end of the tubing was flanged by pressing it against a moderately hot metal surface. A small hole was then cut in the side of the tubing about 2 cm from the nonflanged end. Using a No. 3 cork borer, a small disc of about 0.5 cm in diameter was cut from a sheet of Silastic^{R} surgical plastic, and a hole was cut in the center of this disc with a No. 2 cork borer. The result was a small ring which fit snuggly around the cannula. Two of these rings were prepared for each bladder cannula. The cannula was threaded through one of the rings and the ring was secured to the flanged end of the tubing with epoxy resin. The other ring was placed on the cannula, but not permanently secured.

D.) Animal Clippers - An Oster model A-5 Animal Clipper was used for all presurgical shavings.

E.) Surgical Supplies - Various general surgical instruments were necessary for this study such as assorted scissors, dissecting and tissue forceps - both blunt and pointed, and several straight and curved hemostats. Two types of suture were needed: 4-0 cotton, and 40-cotton Cord. Both types of suture were obtained from Ethicon. Two sizes of suture needles were needed, one size for cutaneous suturing and a small noncutting needle for work on the urinary bladder. The needle for suturing the skin and peritoneum was a 3/8 circle with a cutting edge (Anchor Brand 1822-18). The other needle used for putting a purse-string suture in the urinary bladder was a 1/2 circle taper point (Anchor Brand 1842-22). In addition to the above, a self returning Weitlaner type retractor with 3 x 4 blunt prongs and a Crile-Wood Needle Holder with a boxlock were necessary for the required surgery.

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F.) Infusion Pumps - Two compact Havard model 975 Infusion Pumps were used.

Procedures:

Following anesthesia with an intraperitoneal injection of 50 mg/kg of sodium pentobarbital (Nembutal^R), the rats were shaved on the ventral side of the neck, on the head between the ears, and on the abdomen from the level of the diaphragm to the penis.

A median ventral incision was made in the neck as described previously, and the fat and fascia were cut medially with care taken not to damage the parotid or submaxillary glands. The glands and fat were retracted laterally to expose the sternohyoid muscles. With curved blunt forceps, the left sternohyoid muscle was displaced medially and the carotid artery was located dorsal to it and medial to the omohyoid. With closed blunt forceps, the nerves which run along the carotid artery were separated from the artery. A ligature was then passed under and around the artery, taking care to exclude the nerves. This ligature was tied as close to the head as possible. A second ligature was passed under the artery, but not tied. Stretching the artery with closed blunt forceps to stop blood flow, a small incision was made in the arterial wall with a pair of microdissecting scissors. The cannula was then inserted slowly into the artery through the incision, up to the collar on the cannula. The collar was placed on the cannula at a distance from the end which would allow the tip of the cannula to be in the aorta when the cannula was secured in place. The untied ligature was next tightened and tied around the cannulated artery. A second ligature was then tied around the artery and the cannula was exteriorized through the skin between the ears in the same manner as was described previously for the jugular cannula.

The bile duct was cannulated by the method of Knapp, Lesson, and Wright (30). The main deviation from their method was that a smaller size tubing was used. Whereas they used P.E. 50 for a bile cannula, P.E. 10 was used here.

For implantation of the cannula in the urinary bladder, a midline incision was made in the lower abdomen. The opening was enlarged with a Weitlaner type hand retractor to enable location of the bladder and to provide room for surgical manipulations. The apex of the bladder was carefully grasped with blunt forceps and the urine was withdrawn using a syringe with a 26 gauge needle. A purse-string suture of number 4-0 cotton was sewn around the bladder, about 0.5 cm from the apex. It was necessary to use a 1/2 circle, taper point needle for this procedure in order to avoid severely damaging the bladder wall. With microdissecting scissors, the bladder was cut at the apex, and the opening was enlarged by stretching with an opened blunt forceps. The flanged end of the cannula was then inserted into the bladder, and the purse-string was tightened and tied securely. Following suturing of the peritoneum and the abdominal musculature, a second ring of surgical plastic was placed around the cannula and pressed firmly against the exterior side of the abdominal incision. This stabilized the cannula and secured the bladder against the inside of the abdominal wall. Following completion of the surgery, the bladder was washed with normal saline until no blood was observed in the washings.

The skin was then sutured and the rat was placed in a restraining cage. The carotid cannula was connected to 2 Compact Harvard Infusion Pumps by means of a Travenol four-way stopcock fitted with a 26 gauge

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needle. One pump was used for infusing tritiated methotrexate, and the other for the infusion of probenecid. Loading doses of both drugs were given at a flow rate of 10 ml/hr, until the desired amount of drug had been administered, and then constant infusions were maintained at flow rates of 1.2 ml/hr.

Bile and urine samples were collected over intervals of 1 hour. These were collected directly into dialysis casing sacks from the cannulae. The bladder was washed at the end of each hourly sample period by injecting 2 ml of water into it. This was accomplished by means of a syringe to which was connected a 10 cm length of P.E. 20 tubing. This tubing was inserted into the bladder via the hole which had been cut into the side of the cannula. This 2 ml washing was collected and dried with the previous hourly urine sample. The bile and urine samples, following drying, were oxidized and the radioactivity present was determined as described under Assay Techniques.

Blood samples were withdrawn at the midpoint of each bile and urine collection period. At specified times 0.5 ml of plasma was obtained, which was assayed for probenecid.

Transport Kinetic Studies

Materials:

The materials necessary for these studies were the same as those needed for the Clearance Study.

Procedures:

Rats were surgically prepared in the manner described for the Clearance Study. Following preparation, the rats were placed in restraining cages and allowed to recover from anesthesia for about an hour.

Two sets of infusion studies were conducted in this series. One group of rats, following a loading dose, was infused with only tritiated methotrexate for 8 hours. Blood, bile, and urine samples were collected. The second group was initially given a loading dose of probenecid followed by a constant infusion of this drug. After the probenecid infusion was started, a loading dose of methotrexate was administered. The 2 drugs were then infused for up to 10 hours. Blood, bile, and urine samples were collected.

Methotrexate Bolus - Probenecid Infusion Studies

Materials:

The materials necessary for these studies were the same as those needed for the Transport Kinetic Studies. Only jugular and carotid cannula were needed, however, as no bile and urine samples were collected.

Procedures:

A loading dose of probenecid was administered intraarterially to rats who had been surgically prepared with jugular and carotid cannulae. Following the loading dose a constant infusion of the drug was begun which was maintained throughout the study. After the probenecid had been infused for 1 hour, a bolus of methotrexate was administered via the jugular cannula. The drug was washed through the cannula with heparinized saline, and the cannula was replugged. Blood samples of about 0.5 ml were drawn at specified times for the next 8 hours. Plasma samples to be assayed for probenecid were taken at 1, 4, and 6 hours after the administration of methotrexate. A plasma sample, to serve as a control for the probenecid assay was also taken before the initial administration of probenecid.

Blood samples were placed in dialysis casing sacks, dried, and oxidized in preparation for scintillation counting.

RESULTS AND DISCUSSION

Toxicity Screening Studies

The objective of these studies was to screen drugs suspected of being capable of inhibiting the biliary secretion of methotrexate. Since the measurement of the biliary secretion of methotrexate is a somewhat complicated procedure, a more simple method was required to facilitate screening these compounds.

The hypothesis is being pursued that the gastrointestinal toxicity of methotrexate is related to the biliary secretion of this compound, and it was reasoned that some clinically observable parameter of gastrointestinal toxicity, if properly quantitated, could serve as an indication of the effect different compounds have on the biliary secretion of methotrexate. Though this approach admittedly necessitates numerous assumptions, it was deemed justifiable in light of the limited objectives of this screening study.

The morphological changes in the intestinal mucosa, following exposure to methotrexate, have been well studied (31,32). Within 6 hours of administration of an adequate dose of methotrexate to rats, interstitial edema of the villi becomes apparent, and by 24 hours the epithelial cells begin to desquamate. Within 72 hours the tips of the villi are denuded, and ulcerative colitis and ileitis are evident. In both man and dogs, hemorrhagic enteritis has been reported (33), but blood in the feces of rats has not been observed.

As a result of massive disruption of the intestinal mucosa, sodium and water pass freely into the lumen, and death from dehydration can occur (30). Clinically, what one observes is massive, often bloody diarrhea accompanied by marked dehydration. With the exception of fecal blood loss, which does not appear to occur in rats, none of the observations associated with methotrexate toxicity are quantifiable. It was decided that the only indicator of methotrexate toxicity which did not require a subjective evaluation was mortality. Sandberg and Goldin (32) had used mortality as an endpoint in studies on the prevention of gastrointestinal toxicity of methotrexate by leucovotin (^{5}N-formyltetrahydrofolic acid), and found this to be an acceptable indicator.

At low doses of methotrexate, one would anticipate that preventing the accumulation of methotrexate in the gastrointestinal lumen, by inhibiting biliary secretion, would result in less mucosal damage. When considering high doses, however, one is not certain what overall effect might be observed. By preventing biliary secretion of methotrexate, one would anticipate that the exposure of the intestinal mucosa to methotrexate from within the lumen would be decreased, but with the primary route of elimination from the blood blocked, the systemic exposure would be greatly increased. One might, therefore, expect to actually observe an increase in the severity of toxicity, as well as the incidence of death, when inhibitors of methotrexate's biliary secretion are administered. Since mortality was the indicator chosen to be measured, it was thus necessary to administer high doses of methotrexate. It was anticipated that groups of rats treated with compounds which inhibit the biliary secretion of methotrexate would have a higher mortality rate than would the control groups, which were injected only with methotrexate.

With a dose equivalent to the LD$_{50}$ it was anticipated that it would

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be possible to detect either an increase or a decrease in the incidence of death due to methotrexate toxicity when drugs suspected of inhibiting methotrexate's biliary secretion were administered concurrently. Ferguson et al. (33) reported an LD_{50} for a single intraperitoneal dose of methotrexate in rats to be 6 to 25 mg/kg. They used Wistar rats, of both sexes, weighing from 80 to 150 grams in their study. Since the LD_{50} of methotrexate was not precisely known, a dose of 12.5 mg/kg was chosen.

Two drugs were selected for initial screening as possible inhibitors of the biliary secretion of methotrexate, probenecid and sulfinpyrazone. One of the first reports that probenecid might affect the biliary transport of other compounds was that of Blondheim (34), who showed that probenecid administration interferred with the bromsulfophthalein (BSP) liver function test. In a later study, Goetzee et al. (35) further investigated this effect of probenecid on BSP uptake by the liver and transport into the bile. They concluded that probenecid reversibly inhibits the uptake and storage of BSP by the liver, as well as the excretion of the dye into the bile. They also found that probenecid is a potent choleretic agent. Hart and Schanker (36), while studying secretion of chlorothiazide into the bile, tested the effect of probenecid on the biliary secretion of this compound. They reported that probenecid significantly inhibited the biliary transport of chlorothiazide, and noted, as had Goetzee, that following administration of probenecid, there was a marked cholersis.

Probenecid appears to be the only compound whose effect on biliary secretion of other drugs has been studied to any significant degree. Despopoulos (19), however, reported a great similarity in the structural characteristics of compounds actively secreted into the urine and bile.

From this observation it was suspected that drugs which inhibit the active transport of compounds in the renal tubule, might also have an inhibitory effect on the biliary transport of the same compounds.

Sulfinpyrazone has been shown to possess a uricosuric effect similar to that of probenecid (37), and it has also been shown that probenecid and sulfinpyrazone appear to interfere with each others excretion (38). Sulfinpyrazone, having a pharmacologic activity similar to that of probenecid and being apparently transported in the kidney by the same systems as probenecid, was chosen as an alternative candidate for initial screening.

Two considerations were taken into account in choosing an appropriate dose of probenecid and sulfinpyrazone. The dose utilized must be substantially below the LD_{50} so that the contribution to mortality from these compounds alone could be neglected, but the dose must be high enough to provide adequate levels of the drug in the body to sufficiently inhibit the biliary transport process. No data on the toxicity of sulfinpyrazone was located, but for an intraperitoneal injection of probenecid to rats, the LD_{50} was reported to be 342 mg/kg (39).

Neff, Tozer, and Brodie (40), in a study on the transport of 5-hydroxyindoleacetic acid across the blood brain barrier, found that a dose of 200 mg/kg of probenecid, administered intraperitoneally, was adequate to significantly inhibit this transport process. A dose of 200 mg/kg was thus chosen for this screening study. Since it was desirable to maintain an inhibitory concentration in the blood for at least a couple of hours, the probenecid was administered as two doses of 100 mg/kg. These were administered 20 minutes before and 20 minutes after the methotrexate was injected. The sulfinpyrazone was arbitrarily administered in a single

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dose of 100 mg/kg.

Since the dose of methotrexate administered was in the range of the reported LD_{50}, it was anticipated that about half of the rats in the control group would die. However, none of the rats in the control, the probenecid treated, or the sulfinpyrazone treated groups died. Tests were conducted daily on feces to detect the presence of occult blood, but no positive results were recorded for any rat during the 30 day observation period. An apparent difference in average weight gain between the different groups, however, was noted (Table II). After being analyzed statistically, these differences were found not to be significantly different. The groups receiving only methotrexate and methotrexate plus sulfinpyrazone showed average weight gains during the 30 day study period of 24.4 and 34.7 grams, respectively. The probenecid treated group gained an average of only 0.8 grams. Since no deaths were observed, the dose of methotrexate was apparently below the actual LD_{50} for an intraperitoneal injection of methotrexate for Sprague Dawley rats. It was decided to increase the dose of methotrexate and repeat the study.

The dose of methotrexate was doubled to 25 mg/kg. Again, no deaths were reported in the control group, but deaths were noted in both the sulfinpyrazone and the probenecid treated groups. Within 4 days of the injections, all the probenecid and sulfinpyrazone treated rats had diarrhea and appeared to have dried blood around the eyes and nostrils. Of those rats which died, none did so prior to the third day following the administration of the drugs, and most died between the fourth and seventh days. Eight out of the 10 probenecid treated rats died; 1 of the sulfinpyrazone treated group died; and no rats receiving only methotrexate died (Table III).

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TABLE II

SCREENING STUDY I - ANIMAL WEIGHT CHANGE DATA

Drugs Administered	N	Average Initial Weight ± S.E.	Average Final* Weight ± S.E.	Average Change ± S.E.
Methotrexate 12.5mg/kg	10	226.3± 4.68	250.7± 12.8	24.4± 13.30
Methotrexate 12.5mg/kg + Sulfinpyrazone 100 mg/kg	10	237.3± 3.09	272.2± 5.14	34.7± 2.70
Methotrexate 12.5mg/kg + Probenecid 200 mg/kg	10	242.7± 4.21	243.5± 9.82	0.8± 9.54

* Final weighing was conducted 30 days after drug administration.

TABLE III

SCREENING STUDY II - ANIMAL MORTALITY

Drugs Administered	Number of Deaths 0 - 5 days	5 - 10 days	Total*[a]
Methotrexate[b] 25 mg/kg	0	0	0/10
Methotrexate 25 mg/kg Sulfinpyrazone 100 mg/kg	1	0	1/10
Methotrexate 25 mg/kg Probenecid 200 mg/kg	4	4	8/10

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[a] *Animals were observed for 30 days after drug administration.

[b] Administered as a single intraperitoneal injection.

[c] Administered as a single intraperitoneal injection twenty minutes before the methotrexate was administered.

[d] Administered as two intraperitoneal injections of 100 mg/kg each. One was administered twenty minutes before and the other twenty minutes after the methotrexate was administered.

Methotrexate Bolus Studies - Effect of Probenecid

The Toxicity Screening Studies established that the concurrent administration of probenecid and methotrexate results in an interaction between these compounds. It had been assumed that this interaction was taking place in the liver where probenecid was inhibiting the biliary secretion of methotrexate. Since biliary secretion is the major process by which methotrexate is eliminated from the blood, this inhibition should result in prolonged and elevated blood levels of the drug.

The intent of this study was to determine how significantly the blood level-time course of methotrexate in rats was affected by the concurrent administration of probenecid. These studies were conducted in a manner almost identical to the procedure which was followed for the second Toxicity Screening Study. Following intravenous administration of tritiated methotrexate to rats, the concentration of drug in whole blood was determined as a function of time. In these studies the total blood concentration of tritium label was assumed to reflect methotrexate concentration. The separation of tritiated methotrexate from other tritiated species in the blood is discussed in a later section.

Since the intent of this study was only to show whether or not the elimination of methotrexate from the blood was being significantly inhibited by probenecid, no kinetic analysis of the blood level data was performed. Figure 11 illustrates a semilogarithmic plot of the blood level-time course of methotrexate in rats which were injected with methotrexate alone. Each point represents the geometric mean of the blood level from 5 different rats. The brackets indicate the standard error of each point. The rapid decline of the blood level of methotrexate dur-

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FIGURE 11. Semilogarithmic plot of methotrexate blood concentration with time in rats which were intravenously injected with 25 mg/kg of the drug. The data for this plot are given in Table IV.

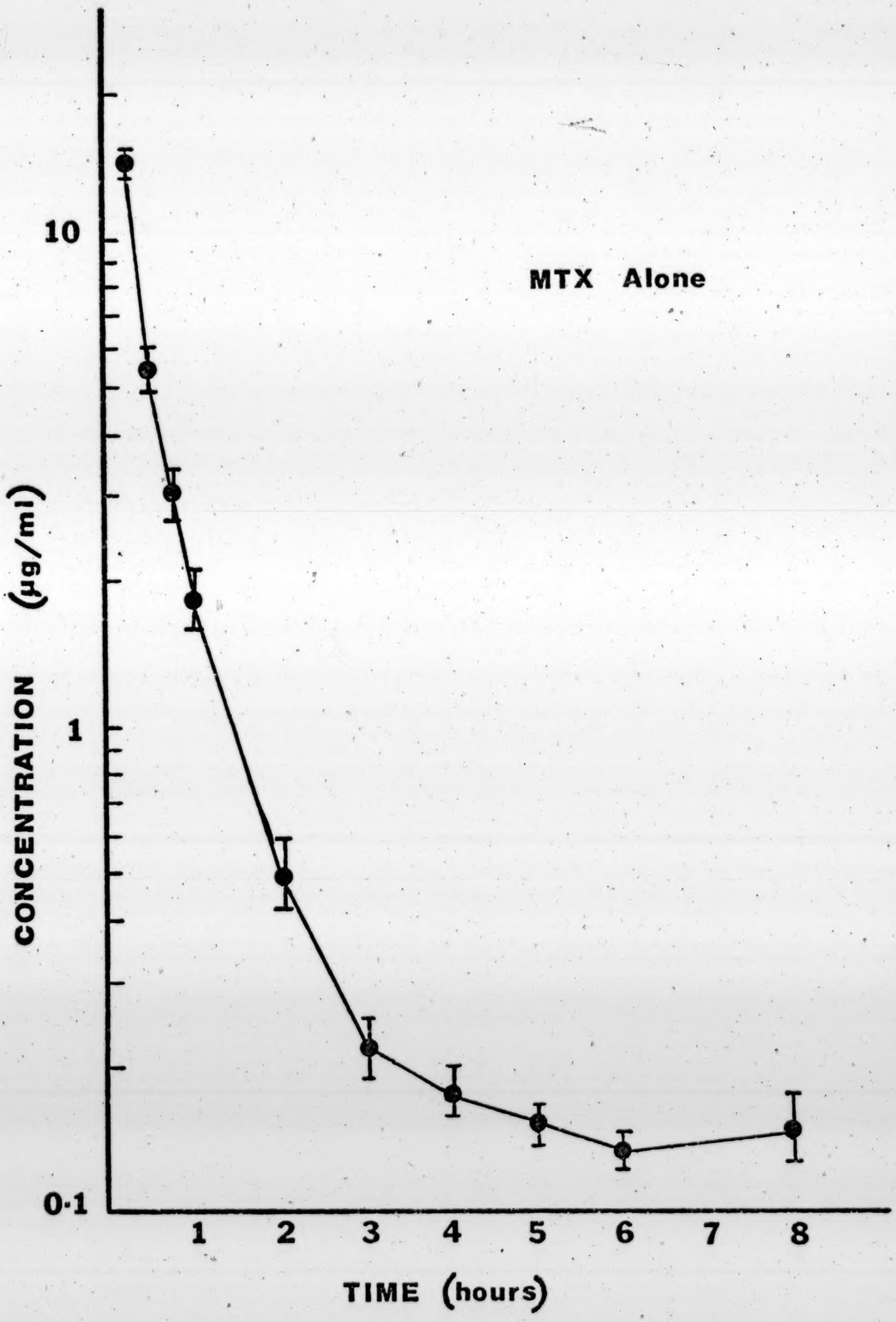

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TABLE IV

BLOOD CONCENTRATION-TIME DATA FOR A SINGLE I.V. INJECTION OF 25 MG/KG OF METHOTREXATE

Sample Time (hours)	Methotrexate Concentration* (mcg/ml)	Log of Methotrexate Concentration	Standard Error of the log of Concentration
0.25	14.9	1.173	$2.63X10^{-2}$
0.50	5.51	0.741	$4.45X10^{-2}$
0.75	3.03	0.481	$5.58X10^{-2}$
1	1.84	0.265	$6.11X10^{-2}$
2	0.500	-0.301	$7.26X10^{-2}$
3	0.218	-0.662	$6.25X10^{-2}$
4	0.177	-0.752	$5.15X10^{-2}$
5	0.153	-0.815	$3.54X10^{-2}$
6	0.133	-0.876	$3.57X10^{-2}$
8	0.150	-0.824	$6.50X10^{-2}$

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*The methotrexate concentrations given are the geometric mean of the concentrations determined in five different rats. The data for the individual rats are presented in Appendix I.

ing the first 2 hours was believed to be due to rapid biliary secretion of the drug. It was, therefore, anticipated that this rapid initial decline would be absent if probenecid were administered, assuming that the effect of probenecid was on the biliary transport process. This is precisely what was observed.

Whereas the blood level of methotrexate in the nonprobenecid treated rats declined to around 0.5 micrograms/ml in the first 2 hours following administration, in the probenecid treated group it took 6 hours before the blood level had declined to this level. Figure 12 illustrates a semilogarithmic plot of the geometric means of the blood levels obtained from the probenecid treated rats. In this group, the blood level of methotrexate appears to decline monoexponentially with a half life of about 1.25 hours in the period of 1 to 6 hours. Since the probenecid blood levels are also declining during this time period, no kinetic significance can be given to this apparent first order process. The blood levels of methotrexate in the probenecid treated rats, between 1 and 8 hours were markedly greater than the levels at the corresponding times in the nonprobenecid treated rats. The blood concentrations in the probenecid treated rats were 4.5, 12, and 2.7 times the levels in the nonprobenecid treated group at 1, 3, and 8 hours, respectively.

This study clearly demonstrated that probenecid inhibits the elimination of methotrexate from the blood. If it is assumed that only linear, first order, processes are involved in the elimination of methotrexate, an estimate of the effect of probenecid by this regimen on the total body clearance of methotrexate can be made by comparing the area under the blood concentration-time curves (AUC) for methotrexate in the presence and absence of probenecid. It will be shown later that at high blood

FIGURE 12. Semilogarithmic plot of methotrexate blood concentration with time. Rats were injected intravenously with 100 mg/kg of probenecid 20 minutes before and 20 minutes after the injection of 25 mg/kg of methotrexate. The data for this figure are given in Table V.

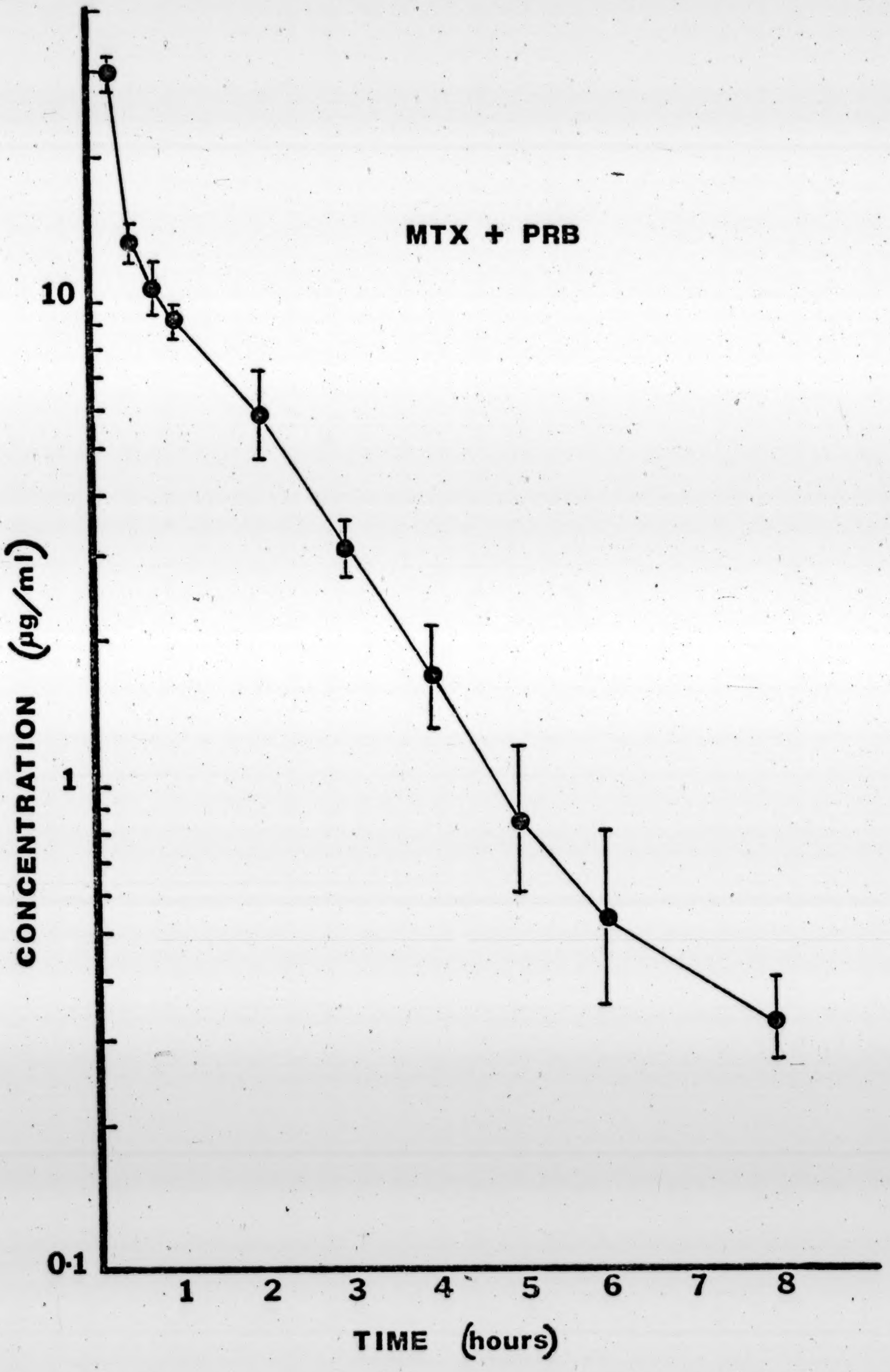

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TABLE V

BLOOD CONCENTRATION-TIME DATA FOR A SINGLE I.V. INJECTION OF 25 MG/KG OF METHOTREXATE ADMINISTERED CONCOMITANTLY WITH TWO 100 MG/KG DOSES OF PROBENECID

Sample Time (hours)	Methotrexate Concentration* (mcg/ml)	Log of Methotrexate Concentration	Standard Error of the log of Concentration
0.25	29.5	1.47	$3.45X10^{-2}$
0.50	13.2	1.12	$4.11X10^{-2}$
0.75	10.7	1.03	$5.48X10^{-2}$
1	9.07	0.958	$3.83X10^{-2}$
2	5.81	0.764	$9.26X10^{-2}$
3	3.12	0.494	$6.00X10^{-2}$
4	1.70	0.230	$1.08X10^{-1}$
5	0.84	-0.076	$1.56X10^{-1}$
6	0.53	-0.276	$1.77X10^{-1}$
8	0.33	-0.481	$8.53X10^{-2}$

[*] Methotrexate concentrations are the geometric means of data from 5 different rats. The data for the individual rats are presented in Appendix I.

concentrations of methotrexate saturation of biliary secretion does occur, but at the low levels of methotrexate observed in this study, linear kinetics is expected. Using the trapezoidal rule (41), the areas under the two blood concentration-time curves were determined for the time period 15 minutes to 8 hours. When methotrexate was administered alone, the area for the 25 mg/kg dose was 6.4 microgram-hours/ml; when administered with probenecid, the area was found to be 27.5 microgram-hours/ml. Since total body clearance is related to area by the equation:

$$\frac{\text{DOSE}}{\text{AREA}} = \text{Total Body Clearance,} \qquad \text{(Eq. 1)}$$

it is apparent that the total body clearance of methotrexate was reduced to approximately 25% of normal by the administration of probenecid. The dose of methotrexate in both cases was the same.

Methotrexate is not significantly metabolized, and in the rat, biliary clearance is the primary component of total body clearance, with renal clearance accounting for between 30 and 40% of total body clearance (13). A reduction of the total body clearance of methotrexate by probenecid to 25% of normal would, therefore, require that probenecid significantly inhibit the biliary secretion of methotrexate. Even if probenecid were to completely inhibit renal clearance, the total body clearance of methotrexate would be only reduced to 60% of normal. Since probenecid only effects the active component of renal secretion, and has no effect on the filtration component, the actual reduction in total body clearance which can be attributed to a decrease in renal secretion is very small in comparison to the observed decrease in total body clearance. Since biliary secretion is the other major component of the total body clearance of methotrexate, these data strongly suggest that probenecid inhibits the biliary secretion of methotrexate.

Separation of Methotrexate From Nonmethotrexate Components in Plasma

Metabolism of methotrexate by rabbits, pigeons, and guinea pigs has been well established (42,43,44). There is some controversy, however, over whether or not methotrexate is metabolized by either rat or man. There is some evidence to suggest that while rat or man may not metabolize methotrexate, it may be metabolized by their intestinal bacteria. Henderson (13,14) was one of the first to investigate the fate of tritiated methotrexate following both oral and parenteral administration in different species. He included in his studies a variety of rodents, as well as primates and man. Following administration of tritiated drug to these species, he collected both urine and bile and separated the labeled components in these samples by column chromatography using diethylaminoethylcellulose. He found that 95 to 100% of the tritium label eluted as a single peak, suggesting no significant metabolism had occurred. However, if urine or bile samples were kept for over four days before being chromatographed, nonmethotrexate labeled species appeared. He attributed this appearance of metabolites or decomposition products to metabolism of the drug by bacteria present in the samples. He also found no metabolism of methotrexate occurring when he conducted a study *in vitro* using the 9000XG supernatant fraction of rat liver homogenates. His conclusion was that methotrexate was not significantly metabolized by these species, but his data did suggest that bacteria could metabolize this compound.

A few years after Henderson reported his work, two communications (45,46) appeared which disputed his findings in regard to the metabolism of methotrexate in mice and rats. The data reported in these communications did not justify any conclusions that these animals could significantly metabolize methotrexate, but it was shown that nonmethotrexate labeled compounds were present to varying degrees in feces and urine samples of animals to whom tritiated drug had been administered. Whether or not the possibility of bacterial metabolism was precluded by the experimental conditions was not reported. Unfortunately, neither of the communications adequately reported the experimental techniques used, and thus the author's interpretation of the data cannot be fully evaluated.

Valerino et al. (47) have recently reported the separation of at least 3 metabolites following incubation of tritiated methotrexate with caecal contents obtained from mice. Following incubation for 6 hours, 76% of the recovered radiolabel was found to migrate more rapidly than methotrexate on a thin-layer chromatographic system using 0.1 M ammonium bicarbonate buffer (pH 8.3) as solvent. They tentatively identified one of these metabolites as 4-amino-4-deoxy-^{10}N-methylpteroic acid (APA) from its ultraviolet and infrared absorption spectra.

In an effort to establish the role played by the bacteria in the metabolism of methotrexate, Valerino also incubated tritiated methotrexate with the caecal contents from mice whose gut lumen had been partially sterilized by pretreatment with neomycin and sulfathiazole for 5 days. In this case, the percent of recovered label which was found to be associated with methotrexate metabolites was only 11, a considerable reduction from the 76% found when no pretreatment was performed.

When tritiated methotrexate was incubated with homogenates of caecal tissue which had been washed free of all contents, no metabolism was found to occur. Their conclusion from these data was that methotrexate was significantly metabolized by the bacteria normally found in the caecum of mice.

Valerino (48) also reported the separation of 4 different metabolites in the urine and feces of rats receiving tritiated methotrexate by gastric incubation, and tentatively identified 2 as 2,4-diamino-6-carboxylpteridine and 2,4-diamino-6-methylpteridine. In this study, Valerino was careful to show that the metabolites produced were formed in vivo, and not as a result of breakdown of methotrexate during the extraction and separation procedures, nor due to bacterial metabolism after the samples were collected. Though the possibility that methotrexate undergoes some degradation in the stomach (49) is considered by Valerino, he suggests that the primary source of methotrexate metabolism is the bacteria of the lower intestine.

Although metabolites of methotrexate have been demonstrated in the urine and feces of animals, their presence in the blood has not been established. It was therefore undertaken to establish whether these metabolites appear in the blood and to devise a means for separating tritiated methotrexate from other labeled species in blood. In the previously discussed Methotrexate Bolus Studies the blood levels of radiolabel were assumed to represent unchanged drug. However, for more quantitative work, corrections for the presence of radiolabeled species other than methotrexate must be made.

As a preliminary study, purified tritiated methotrexate was added to rat whole blood, extracted, and chromatographed. The objectives were to determine whether there was any degradation of methotrexate occuring during the extraction or chromatographing procedures, and to establish an Rf value for methotrexate in this system. The chromatogram obtained from this study (Fig. 13) contained only 1 peak, indicating that methotrexate was not degraded during the extraction or chromatographing procedures. This peak had an Rf value of 0.5, which is in good agreement with an Rf of 0.56 reported by Valerino (47) in a similar chromatographic system.

The chromatograms of plasma samples taken from rats within the first hour after injection of tritiated drug contained only 1 peak. However, in samples taken from 1 to 6 hours after injection, 2 peaks were observed. Figure 14 illustrates a chromatogram from a plasma sample taken from a rat 2 hours after injection of the labeled drug. The Rf value of this pre-methotrexate peak was found to be 0.23. Using this same system, Valerino (48) found 4 different metabolites in the urine and feces of rats to migrate with an Rf value of 0.26 to 0.27.

The percent of total label recovered which was not associated with methotrexate increased with time (Table VI). Though the percentage of label which was associated with the nonmethotrexate components increased with time, this did not necessarily indicate that the amount of the nonmethotrexate component was increasing. By multiplying the plasma concentration of total radiolabel in the plasma by the percent of label under the methotrexate peak, the actual plasma level of methotrexate could be obtained. As shown in Fig. 15, the actual concentration of the nonmethotrexate component appeared to remain relatively constant, though the percentage of total label represented by this component did increase.

If the nonmethotrexate component exists as a trace impurity in the injectable solution, it represents less than a half of a percent of the total radiolabel present. If it is assumed that the nonmethotrexate component is administered as a radiolabeled impurity, the data can be readily explained. Following injection, methotrexate is rapidly eliminated from

FIGURE 13. Chromatogram prepared following extraction of purified tritiated methotrexate from rat blood. An Rf value of 0.5 was recorded for the unchanged methotrexate.

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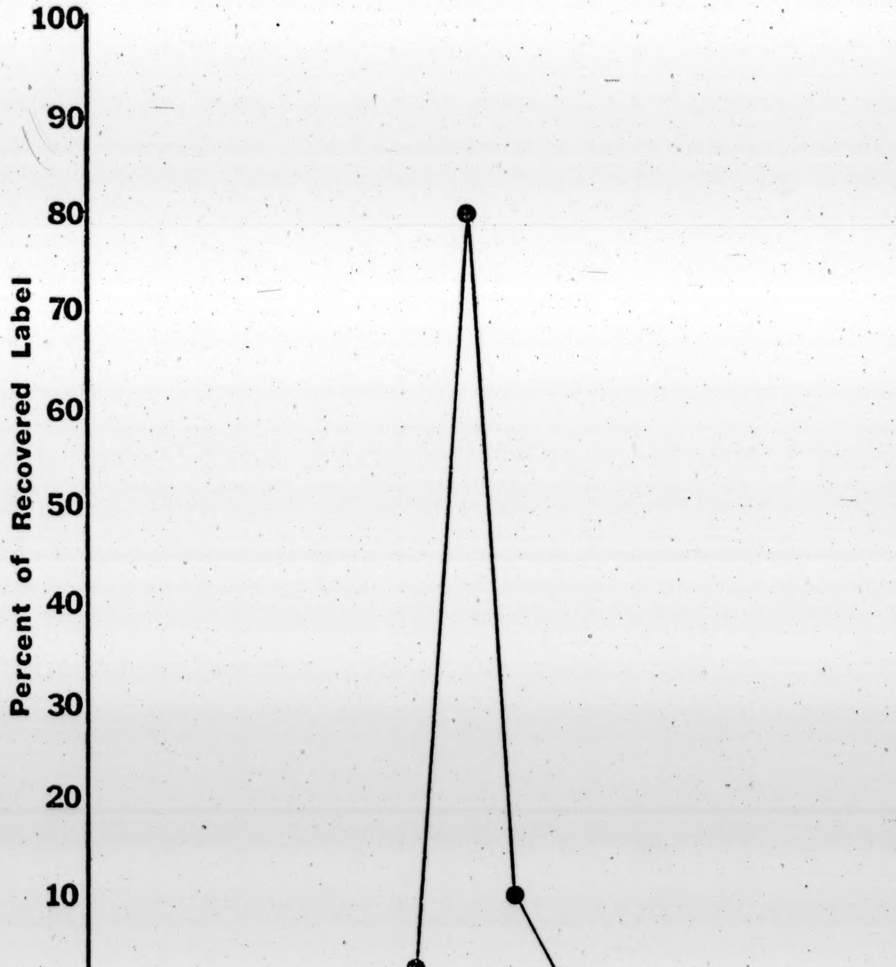
100
90
80
70
60
50
40
30
20
10
Percent of Recovered Label
2
4
6
8
10
12
14
16
Strip Number

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TABLE VI

PERCENTAGE OF TOTAL LABEL IN PLASMA ASSOCIATED WITH METHOTREXATE AND NONMETHOTREXATE PAPER CHROMATOGRAPHIC PEAKS AT VARIOUS PLASMA SAMPLING TIMES*

COMPOUND	PERCENTAGE AT			
	1hr.	2hr.	4hr.	6hr.
Methotrexate (Rf 0.5)	95	87	72	49
Nonmethotrexate (Rf 0.23)	2	5	25	50
Total	97	92	97	99

* Following a 25 mg/kg intravenous dose.
The data for this table may be found in Table II-3, Appendix II.

FIGURE 14. Chromatogram prepared following the extraction of methotrexate and nonmethotrexate labeled components from a plasma sample taken from a rat, 2 hours following the injection of the tritiated drug. As in Fig. 13, an Rf value of 0.5 was recorded for the unchanged methotrexate, and an Rf value of 0.23 was recorded for the nonmethotrexate component.

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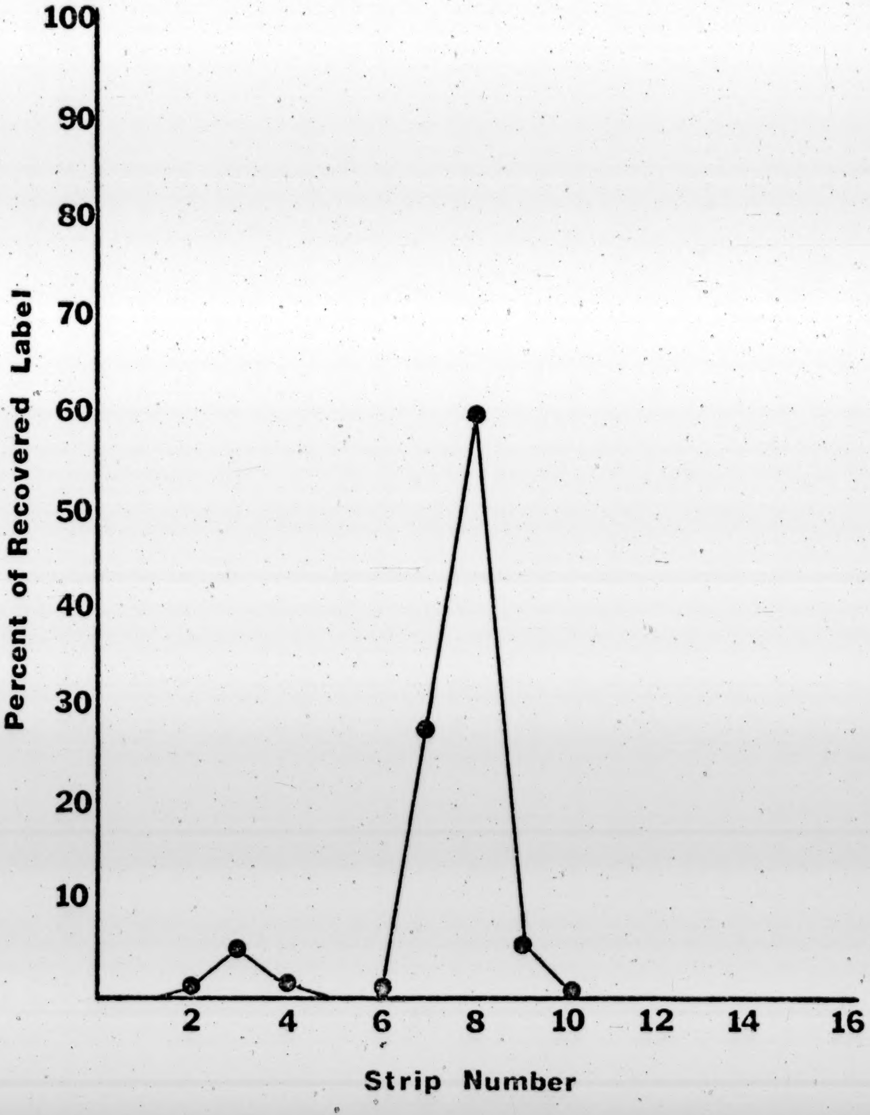
Percent of Recovered Label
100
90
80
70
60
50
40
30
20
10
2
4
6
8
10
12
14
16
Strip Number

FIGURE 15. Plasma concentrations of tritiated methotrexate equivalents with time. Key: ● , Total; ▲ , methotrexate; ■ , nonmethotrexate. The data for this figure may be found in Table II-1, and Table II-2 of Appendix II.

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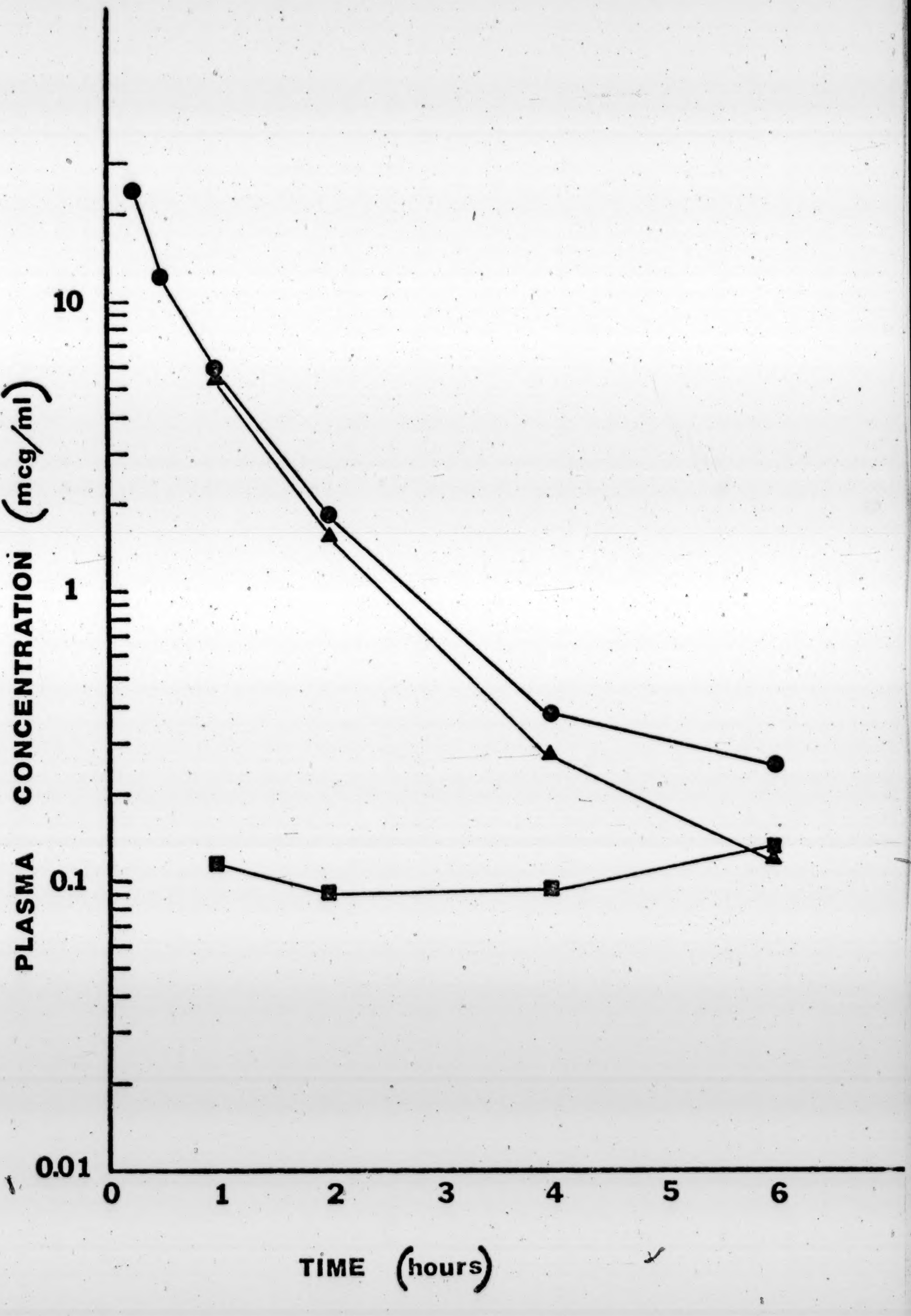

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the blood, and the nonmethotrexate component, being very slowly removed, becomes increasingly the dominant species present. This nonmethotrexate component gains significance with time due to its retention in the blood. Under conditions where a constant level of methotrexate is maintained in the blood by a steady state infusion, no significance need be attributed to this nonmethotrexate component, unless the infusion is maintained for an extremely long time, thus allowing accumulation of this slowly removed impurity.

Another, though less satisfactory, explanation of the data is that methotrexate is metabolized in the intestinal lumen by bacteria, and the nonmethotrexate component in the blood is due to the presence of an absorbed metabolite. Whether the nonmethotrexate component originates from metabolism of methotrexate by intestinal bacteria, from a trace contaminant in the "purified" tritiated methotrexate solution, or from both of these sources is at present unknown (50).

The presence of this nonmethotrexate component in the blood of rats used for the Methotrexate Clearance Study, the Transport Kinetics Studies, and the Methotrexate Bolus-Probenecid Infusion Studies was not considered significant and therefore was not determined. In both the Clearance Study and the Transport Kinetic Studies constant blood levels of methotrexate were maintained by steady state infusions while the bile and the urine were collected. If the nonmethotrexate component is an impurity in the injectable solution, its presence can be ignored for the reason discussed previously. The possibility of metabolism by bacteria in the intestinal lumen can also be discounted as the rats had their bile ducts cannulated, and therefore methotrexate was not available in the intestinal lumen for bacterial metabolism. As a check for the presence of either metabolites

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or impurities, a bile and urine sample taken from a rat at the end of one of these infusion studies were chromatographed. Only 1 peak was observed for each sample. The Rf value of this peak corresponded to that of methotrexate.

The presence of nonmethotrexate components in the blood of rats used for the Methotrexate Bolus-Probenecid Infusion Studies was also not considered significant for reasons similar to those mentioned above. The transport of methotrexate into the intestinal lumen was inhibited by probenecid, and thus the possibility of metabolism by the intestinal bacteria was considered to be very slight. The elimination of methotrexate from the blood was also greatly inhibited in these studies, and therefore the presence of a trace impurity in the injectable solution would not be a significant factor. Only at the later time points, when the level of methotrexate in the blood had been significantly reduced, would the contribution of the nonmethotrexate component to the total radiolabel in the blood be of some importance. However, since the determination of the area under the blood concentration-time curve was the major objective of this group of studies, the significance of the later time points was not considered very great, as almost all the area under the curve can be accounted for in a short time following administration of the drug.

Methotrexate Clearance Study - Effect of Probenecid

The objective of this study was to establish, by direct measurement, that probenecid's primary effect was the inhibition of the biliary secretion of methotrexate. Some inhibition of renal secretion was also expected, and therefore both urinary and biliary clearances were determined. Since clearance components are additive, and their sum equals total body

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clearance, it was anticipated that the sum of renal (Cl_u) and biliary (Cl_b) clearances would equal total body clearance (Cl_{tb}).

$$Cl_u + Cl_b + Cl_{tb} \qquad \text{(Eq. 2)}$$

A large difference between the sum of renal and biliary clearances and the value of total body clearance would suggest that there is another major route of elimination of methotrexate.

In this study a steady state blood concentration of methotrexate was maintained by a constant infusion of the drug. At steady state, the total body clearance of a drug is related to its infusion rate (Ro) and its steady state blood concentration ($C_{b_{ss}}$) by the equation:

$$Ro = C_{b_{ss}} \text{ X } Cl_{tb}. \qquad \text{(Eq. 3)}$$

By rearranging to solve for total body clearance, Eq. 4 is obtained.

$$Cl_{tb} = \frac{Ro}{C_{b_{ss}}} \qquad \text{(Eq. 4)}$$

Both bile and urine were collected during intervals of 1 hour, throughout the study, and the excretion rate of methotrexate, by each route was determined for each sampling interval. At the midpoint of each collection period, a blood sample was obtained and the concentration of methotrexate determined. From these data, the renal and biliary clearances were calculated. Since renal and biliary clearances are the proportionality factors between blood concentration and renal and biliary excretion rates, respectively, these clearances could be calculated from the following equations:

$$Cl_u = \frac{\dot{A}e_u}{C_b} \qquad \text{(Eq. 5)}$$

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$$Cl_b = \frac{\dot{A}e_b}{C_b} \qquad \text{(Eq. 6)}$$

Where,

Cl_u = The urinary clearance

Cl_b = The biliary clearance

C_b = The blood concentration

$\dot{A}e_u$ = The renal excretion rate

$\dot{A}e_b$ = The biliary excretion rate

In order to obtain an accurate estimate of methotrexate clearance by these two routes, steady state conditions were applied. To lessen the time necessary to reach a steady state blood level of methotrexate, a loading dose of 0.5 mg was administered at the start of the study. The drug was infused at a rate of 0.5 mg/hr throughout the study period. The blood concentration-time course of methotrexate is illustrated in Fig. 16. An average blood concentration of 0.78 micrograms/ml was observed during the first five hours of the experiment. At the fifth hour, a loading dose of 15 mg of probenecid was administered and an infusion of 5 mg/hr was maintained for the remainder of the study. As can be seen, following the administration of probenecid, the blood concentration of methotrexate increased significantly.

The continually increasing blood concentration of methotrexate suggests that a steady state level of methotrexate was not reached during the experimental time period. The actual steady state blood concentration can be calculated by rearranging Eq. 3 to give

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FIGURE 16. Blood concentration-time course of methotrexate. Following a loading dose of 0.5 mg, methotrexate was infused at a rate of 0.5 mg/hr. Starting at the fifth hour probenecid was infused at a rate of 5 mg/hr. A loading dose of 15 mg of probenecid was administered at the start of the infusion. The weight of the rat used for this study was 403 gm.

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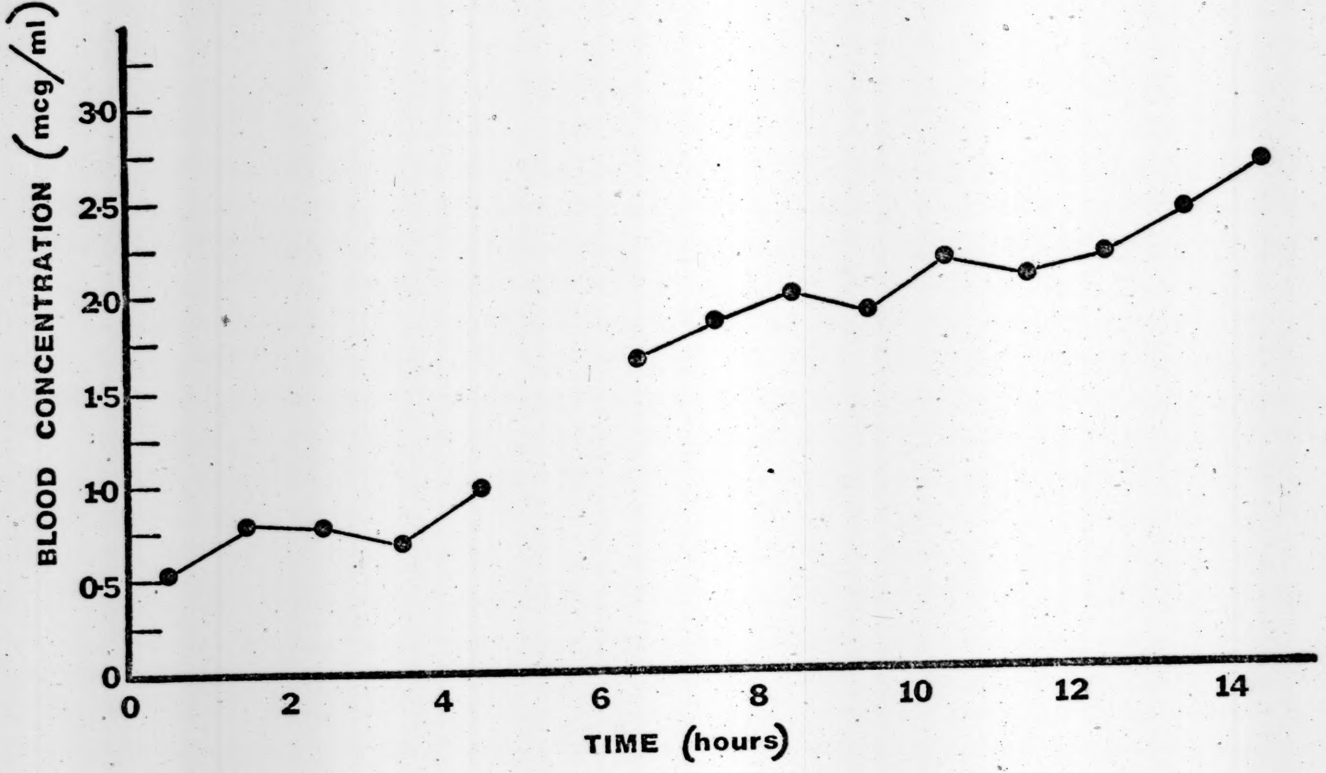
BLOOD CONCENTRATION (mcg/ml)
3·0
2·5
2·0
1·5
1·0
0·5
0
0
2
4
6
8
10
12
14
TIME (hours)

$$C_{b_{ss}} = Ro / Cl_{tb} \qquad \text{(Eq. 7)}$$

Using the sum of the average renal and biliary clearances, obtained during the probenecid treatment period, for total body clearance, a steady state blood concentration of 2.14 micrograms/ml is calculated. This is not significantly different from the average blood concentration (2.12 micrograms/ml) measured during this time interval. A possible explanation for the increasing concentration of methotrexate in the final few time points is that the animal under study is showing some effect of the long study period, or perhaps an increase in the infusion rate due to some idiosyncracy of the infusion pump.

Figure 17 illustrates the biliary and urinary clearance data obtained from this study. The clearance values are plotted at the midpoint of each collection interval. The values for renal and biliary clearance for the first hour are unreliable estimates, and are much higher than the actual clearances. The reason for this error in the first sampling period can be readily explained. The blood concentration drops precipitiously following the initial loading dose, despite the initiation of a constant infusion of the drug. As shown in Fig. 11, methotrexate blood concentrations, following a bolus injection, decreases by at least a factor of 10 during the first hour. The blood concentration which was measured at 30 minutes after the start of the study was too low to be representative of the average blood concentration during this sampling interval. Thus, due to a value for the blood concentration which was too low, the calculated value of clearance was too high.

Ignoring the values for the first hour, the average renal and biliary clearances of methotrexate during the control (1 to 5 hours) period were

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FIGURE 17. Renal and biliary clearances as measured at hourly intervals throughout the study. Methotrexate was infused throughout the study at a rate of 0.5 mg/hr, following an initial loading dose of 0.5 mg. Probenecid was infused from the fifth hour on at a rate of 5 mg/hr, following a loading dose of 15 mg. The rat weight was 403 gm.

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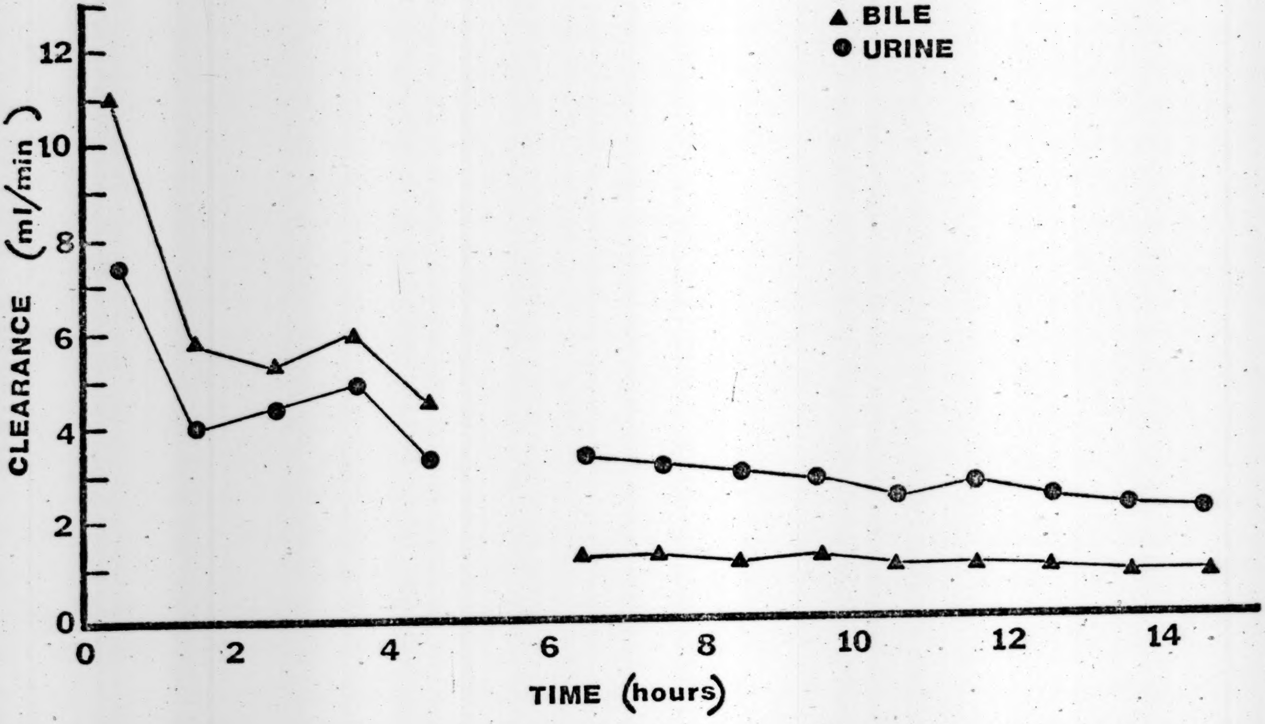
▲ BILE
● URINE
CLEARANCE (ml/min)
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
TIME (hours)

4.3 ml/min., and 5.5 ml/min., respectively. From these data it can be seen that the biliary clearance of methotrexate dropped significantly. The average biliary clearance of methotrexate, between the sixth and the fifteenth hour, was 1.1 ml/min. This was a decrease of 80% from the control value (Table VIII). Whereas during the control period the biliary secretion was the major route of elimination of methotrexate from the blood, during probenecid treatment, the major route of elimination of methotrexate was via the kidneys. It is evident from these data that probenecid exerts its primary effect on the clearance of methotrexate from the blood by inhibiting its biliary secretion.

The inhibitory effect of probenecid on the renal secretion of methotrexate was much less than the effect on biliary secretion. The average renal clearance during the period of probenecid administration was 2.8 ml/min., a decrease of 35% from the control value (Table VII). That methotrexate is actively secreted in the kidney is supported by these data, since probenecid would not be expected to inhibit the filtration component of renal elimination.

The total body clearance of methotrexate, as determined from the infusion rate and the steady state blood concentration, was accounted for, within experimental error, by the sum of renal and biliary clearances (Table VIII). In the control period, the difference between total body clearance and the sum of renal and biliary clearance was about 8%, and for the probenecid treatment period, the difference was around 1%.

Transport Kinetic Studies

The objectives of this group of studies were to characterize the renal and biliary transport of methotrexate, and to quantitatively assess

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TABLE VII

SUMMARY OF DATA FROM METHOTREXATE CLEARANCE STUDY*

	1-5 hours	5-15 hours	Change
Blood Concentration (mcg/ml)	0.78	2.12	172%
Biliary Clearance (ml/min)	5.5	1.1	-80%
Urinary Clearance (ml/min)	4.3	2.8	-35%

* 1-5 hr-control period; 5-15 hr-probenecid treatment

TABLE VIII

COMPARISON OF TOTAL BODY CLEARANCE (TBC) WITH THE SUM OF RENAL AND BILIARY CLEARANCES

	0-5 hours	5-15 hours
Methotrexate Infusion Rate (mg/hr)	0.5	0.5
Average Blood Concentration (mcg/ml)	0.78	2.12
Total Body Clearance (ml/min)	10.7	3.94
Biliary Clearance Plus Renal Clearance (ml/min)	9.8	3.90

the inhibition of these systems by probenecid. The biliary transport mechanism, being the system of primary interest, will be considered first.

The processes involved in the secretion of drugs into the bile have not been as extensively studied, and therefore are not as well understood, as the mechanisms involved in the renal elimination of drugs. Some investigators have speculated that a passive filtration component may play a part in the transport of drugs into the bile (51), but it is generally believed that active transport is the only process involved (52,53,54).

For a drug to be transported from the blood into the bile, it must travel from the liver sinusoid, through the space of Disse, and then through a hepatic parenchymal cell before entering the bile canaliculus. The process by which a drug molecule travels from the sinusoid into the space of Disse is one of simple diffusion. Though the transport from the parenchymal cell into the bile canaliculus is thought to be an active transport process, the nature of the mechanisms involved in the uptake of drugs from the space of Disse by the parenchymal cells is not clearly understood (55). In the past it was believed that uptake was due to physiological binding of drugs to cellular proteins (56), but more recent studies have suggested that an active transport mechanism exists which is responsible for drug uptake by the hepatic parenchymal cells (51,57).

Though the nature of the uptake mechanism is currently an unsettled issue, there is at least one active transport process involved in the secretion of drugs into the bile, this being the transport from the parenchymal cell across the cell membrane into the bile canaliculus. In order for an anionic drug to be secreted into the bile in the rat it must have a molecular weight of 300 or greater (58). Methotrexate, possessing a glutamic acid moiety, and having a molecular weight of 450, satisfies

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this criterion.

The characterization in vivo of the biliary transport of methotrexate was approached in a manner similar to that generally used for the characterization of enzymatic reactions in vitro. As long as the uptake of methotrexate by the parenchymal cell and its transport from the cell into the bile canaliculus occur at greatly different rates, the slower rate limiting, process will be the only one observed kinetically.*

Active transport processes, in general, are subject to saturation, and it was therefore expected that as the blood concentration of methotrexate was increased, the rate of transport of the drug into the bile would approach a maximum, and that the rate of transport could be described by a relationship analogous to the equation of Michaelis and Menten:

$$T = \frac{T_{max}\,(D)}{K_t + (D)} \qquad \text{(Eq. 8)}$$

Where,

T = The rate of transport

T_{max} = The maximal rate of transport

K_t = The transport constant which equals the concentration of drug which results in a rate of transport equal to one half of the maximal rate

(D) = The concentration of drug at the site where transport occurs.

The model, which this equation describes, assumes the formation of a complex (CD) between a transport carrier (C) and the drug (D). This complex is assumed to form in the hepatic parenchymal cell or in the cell membrane. This complex crosses the membrane to deposit the drug into the bile canaliculus; the carrier then returns to the interior of the cell where it complexes with more drug.

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* It has been assumed for purposes of discussion that the transport system involved in the transfer of drug from the parenchymal cell into the bile canaliculus is the rate limiting process.

hepatic parenchymal cell bile canaliculus

$$D + C \rightleftharpoons DC \longrightarrow D$$

The rate limiting step must be neither the association nor the dissociation of the complex, but the translational event separating these events (59).

To adequately characterize this transport mechanism, it is necessary to determine both the value of the maximal rate of transport and the value of the transport constant. This was accomplished by measuring the rate of transport of methotrexate into the bile at various concentrations of the drug. This approach necessitated two assumptions: (1) the concentration of methotrexate in the blood is representative of the concentration at the site of transport, and (2) the rate at which methotrexate appears in the bile duct is equal to the rate of transport into the bile, that is, there is no significant lag time between the time methotrexate is secreted into the bile and the time it can be detected in the bile duct.

In these studies, methotrexate's biliary excretion rate was measured under steady state conditions, and therefore the condition stipulated by the second assumption was controlled.

The data obtained from measuring the rate of biliary secretion of methotrexate as a function of its concentration in the blood can be treated in various ways to determine the values of the transport maxima (T_{max}), and the transport constant (K_t). The Lineweaver-Burk, the Hofstee, and the Woolf rearrangements of the Michaelis-Menten equation are the bases for

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three primary treatments of these type of data.

The Lineweaver-Burk equation is obtained by taking the reciprocal of both sides of the Michaelis-Menten equation. Taking reciprocals of both sides of Eq. 8 and rearranging gives:

$$\frac{1}{T} = \frac{K_t}{T_{max}} \cdot \frac{1}{(D)} + \frac{1}{T_{max}} \qquad \text{(Eq. 9)}$$

A plot of 1/T versus 1/(D), Fig. 18a, gives a straight line with a slope equal to K_t/T_{max} and an intercept on the 1/T axis equal to $1/T_{max}$.

The Hofstee equation is obtained by multiplying both sides of Eq. 8 by (D) and rearranging to yield:

$$T = -K_t \cdot (T/(D)) + T_{max} \qquad \text{(Eq. 10)}$$

A plot of T versus T/(D), Fig. 18b, gives a straight line with a slope equal to $-K_t$ and an intercept on the T axis of T_{max}. The intercept on the T/(D) axis is equal to T_{max}/K_t.

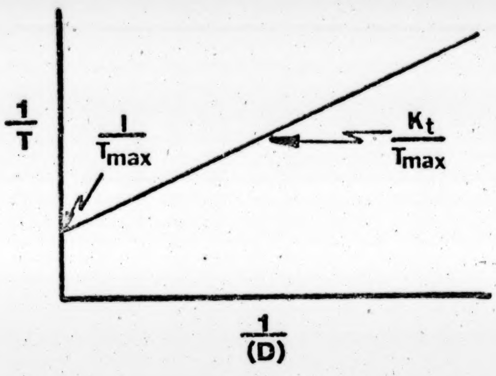
The third method, the Woolf rearrangement of the Michaelis-Menten equation, can be obtained by dividing both sides of Eq. 8 by (D) and taking reciprocals:

$$(D)/T = K_t/T_{max} + (D)/T_{max} \qquad \text{(Eq. 11)}$$

A plot of (D)/T versus (D), Fig. 18c, gives a straight line with a slope of $1/T_{max}$ and an intercept on the (D)/T axis equal to K_t/T_{max}.

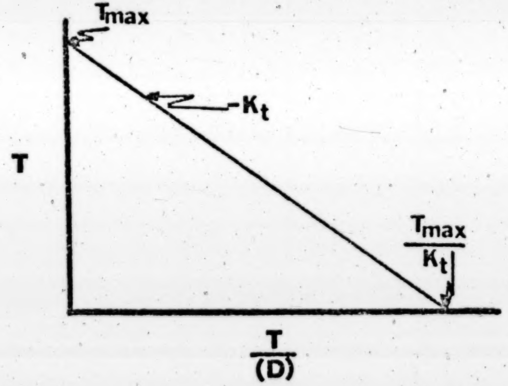
Since Eqs. 9, 10, and 11 are all variants of Eq. 8, it might seem that anyone of these treatments of the data could be used to estimate values of K_t and T_{max} with equal accuracy. This is not the case, however.

FIGURE 18. Linear plots of transport kinetic transport data. (a.) Lineweaver-Burk plot. (b.) Hofstee plot. (c.) Woolf plot.



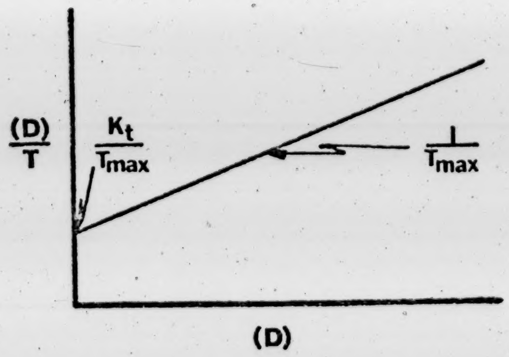
$\frac{1}{T}$
$\frac{1}{T_{max}}$
$\frac{K_t}{T_{max}}$
$\frac{1}{(D)}$

(a)



T_{max}
K_t
T
$\frac{T_{max}}{K_t}$
$\frac{T}{(D)}$

(b)



$\frac{(D)}{T}$
$\frac{K_t}{T_{max}}$
$\frac{1}{T_{max}}$
(D)

(c)

In Eq. 9 and 11, taking the reciprocals of T tends to give unwarranted emphasis to the smallest values of T, and these are the ones which are most likely to have the greatest percentage error in their measurement. In Eq. 10, T appears on both sides of the equation, and thus a plot of T versus T/(D) would inevitably show some degree of correlation. Dowd and Riggs (60) have analyzed these three linear transformations of the Michaelis-Menten equation and have reported that the Lineweaver-Burk method of plotting 1/T against 1/(D) is by far the least reliable method for determining K_t and T_{max}. Plotting (D)/T versus (D), the Woolf plot, was found to be somewhat superior to plotting T versus 1/(D) when the error of T was small, but they found the reverse to be true if the error of T was large. They also reported that a plot of T versus T/(D) often tends to exaggerate deviations from the theoretical relationship and thus is a good indicator that the model may be inappropriate for treating the experimental data. In contrast, the Lineweaver-Burk method may give a good fit to the data, even when the data are unreliable.

The data for the biliary transport of methotrexate were treated by each of the above mentioned methods. The Lineweaver-Burk plot (Fig. 19) gave a good fit to the data ($r = 0.94$). Values for the transport constant (K_t) and the maximal transport rate (T_{max}) were found to be 3.25×10^{-2} mg/ml and 12.09 mg/hr, respectively. The data were fit to equation nine using a least squares fitting program, without any weighting of the data.

The Hofstee plot, Fig. 20, gave a much poorer fit to the data than did the Lineweaver-Burk plot. As reported by Dowd and Riggs, the Hofstee treatment of the data tends to exaggerate deviations from the theoretical relationship. The fit of the data by this treatment was, therefore, not

FIGURE 19. Lineweaver-Burk plot of biliary secretion data. The data for this plot are given in Appendix IV. The values for K_t and T_{max} are given in Table IX. The units of T are mg/hr and the units of D are micrograms/ml.

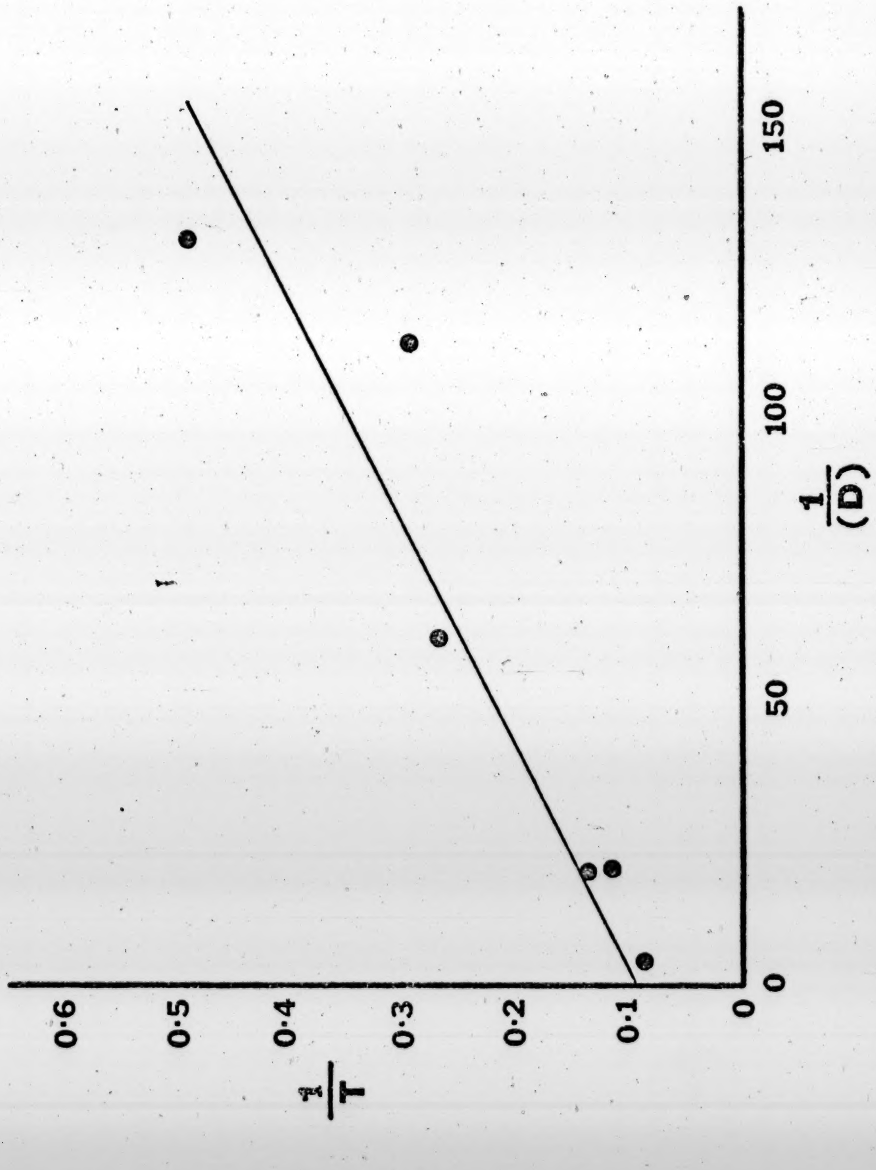


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FIGURE 20. Hofstee plot of biliary secretion data. The data for this plot are given in Appendix IV. The values of K_t and T_{max} are given in Table IX. The units of T are mg/hr, and the units of T/(D) are ml/hr.

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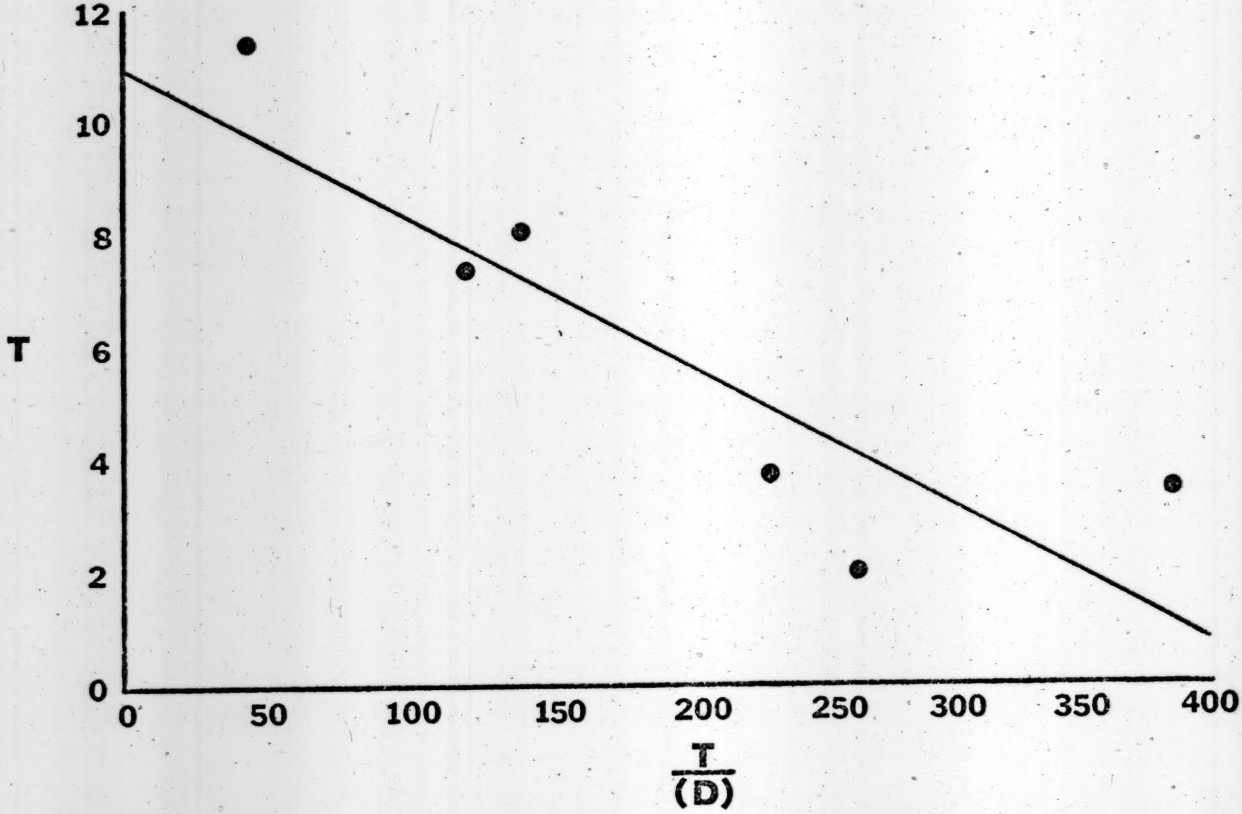

anticipated to be as good as by the other two treatments. A correlation coefficient of 0.858 was obtained when the data were fit to equation 10 using the same nonweighted least squares program as used above. Values of 2.54×10^{-2} mg/ml, and 10.96 mg/hr were obtained for the transport constant and the maximal transport rate, respectively.

The third treatment of the data, the Woolf plot, Fig. 21, gave the best fit to this data ($r = 0.997$). The value of the transport constant was found to be 3.76×10^{-2} mg/ml, and that of the maximal transport rate to be 12.97 mg/hr.

A summary of the values obtained for the transport constant and the maximal transport rate are given in Table IX, along with the correlation coefficient for each plot. The average values obtained for the transport constant and the maximal transport rate from the two "more reliable" plots, the Hofstee and the Woolf plot, are 3.15×10^{-2} mg/ml, and 11.97 mg/hr, respectively. These values are surprisingly close to these obtained from the reportedly "less reliable" Lineweaver-Burk plot.

Having examined the active transport system involved in the secretion of methotrexate into the bile, it was next of interest to consider the inhibition of this system by probenecid.

Inhibition of active transport is generally classified as either competitive or noncompetitive. In the case of competitive inhibition, the inhibitor forms a reversible complex with the transport carrier, and essentially competes with the normal substrate (in this case methotrexate) for these transport carriers. The actual rate at which a drug, or substrate, is transported depends on the relative concentrations of both the substrate and the inhibitor. In the case of noncompetitive inhibition, there is no relationship between the degree of inhibition and the concen-

FIGURE 21. Woolf plot of biliary secretion data. The data for this plot are given in Appendix IV: The values of K_t and T_{max} are given in Table IX. The units of (D) are micrograms/ml, and the units of (D)/T are hr/ml X 10^{-3}.

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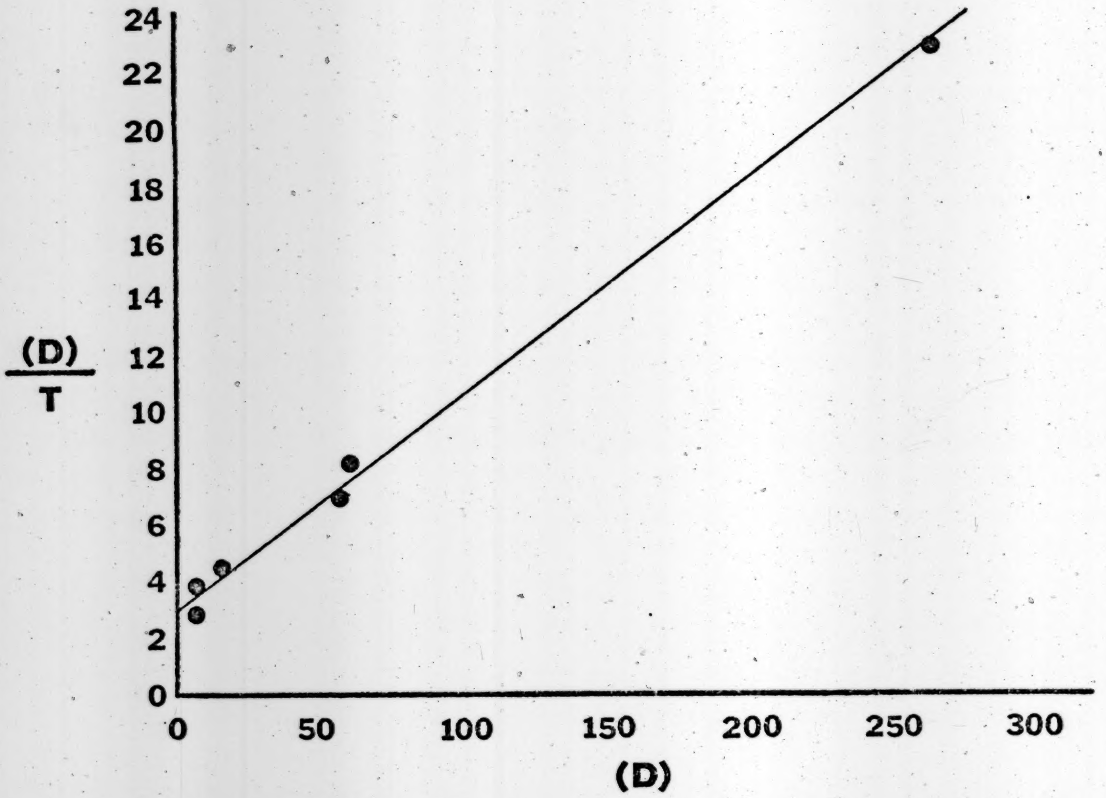

TABLE IX

VALUES OF T_{max} AND K_t FOR THE BILIARY SECRETION OF METHOTREXATE OBTAINED FROM THE LINEWEAVER-BURK, THE HOFSTEE, AND THE WOOLF PLOTS

Parameter	Lineweaver-Burk Plot 1/T vs 1/(D)	Woolf Plot (D)/T vs (D)	Hofstee Plot T vs T/(D)
K_t	0.0325	0.0376	0.0254
T_{max}	12.09	12.97	10.96
r	0.940	0.997	0.858

tration of the substrate. In this case, inhibition depends only on the concentration of the inhibitor (61).

The two relationships which describe systems which are subject to competitive and noncompetitive inhibition are given below:

$$T = \frac{T_{max} \cdot (D)}{K_t(1 + (I)/K_i) + (D)} \qquad \text{(Eq. 12)}$$

and,

$$T = \frac{T_{max} \cdot (D)}{(K_t + (D)) \cdot (1 + (I)/K_i)} \qquad \text{(Eq. 13)}$$

where

(I) = the concentration of the inhibitor

K_i = the dissociation constant of the enzyme-inhibitor complex.

The other symbols are the same as those described previously.

In the case of competitive inhibition, (Eq. 12), the maximal rate of transport is unaffected, and the transport constant is increased by a factor of $(1 + (I)/K_i)$. The effect of a noncompetitive inhibitor, (Eq. 13), is to decrease the maximal rate of transport.

The general protocol followed in examining the inhibition of active transport, requires that the transport rate of a substrate be determined as a function of the substrate concentration, where the concentration of the inhibitor is maintained at a constant level. The treatment of the resulting data by any of the plots in Fig. 18 allows determination as to whether the inhibition is competitive or noncompetitive, as well as the determination of the value of K_i.

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To use this approach in the examination of the inhibition of methotrexate's biliary transport by probenecid, it would be necessary to maintain the same concentration of probenecid in several different rats, while measuring the biliary secretion of methotrexate at various concentrations. The primary drawback to this approach is the difficulty in maintaining the same concentration of probenecid in different rats. It has been shown (62) that the rate of elimination of probenecid in rats, as well as in other species, is subject to large intersubject variations. The difficulties involved in individualizing the rates of administration of probenecid, in order to obtain the same concentration of this drug in each rat, precluded this approach.

The necessity of maintaining the same concentration of probenecid in each rat could be avoided, however, if the studies were conducted at concentrations of methotrexate which were greatly below the value of K_t. If the concentration of methotrexate (D) were much less than K_t, then the concentration of drug can be dropped from the denominator of equation 12 and 13, giving:

$$T = \frac{T_{max} \cdot (D)}{K_t \cdot (1 + (I)/K_i)} \qquad \text{(Eq. 14)}$$

Taking reciprocols of each side of equation 14 and rearranging gives:

$$1/T = K_t/T_{max} \cdot (D) + (K_t/T_{max} \cdot K_i) \cdot (I)/(D) \qquad \text{(Eq. 15)}$$

This relationship makes no assumptions regarding the nature of inhibition, whether competitive or noncompetitive, and can be obtained from either equation 12 or 13. Multiplying each side of equation 15 by (D) gives:

$$(D)/T = K_t/T_{max} + (K_t/T_{max} \cdot K_i) \cdot (I) \qquad \text{(Eq. 16)}$$

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It should be noted that (D)/T is the reciprocol of clearance. A plot of the reciprocol of biliary clearance of methotrexate versus the concentration of probenecid should give a straight line with an intercept on the reciprocol clearance axis of K_t/T_{max} and a slope of $K_t/T_{max} \cdot K_i$. From this plot (Fig. 22) the value of K_i for probenecid can be determined.

To obtain data for this treatment, rats were infused to steady state levels of both methotrexate and probenecid. The biliary secretion rate, as well as the concentration of these 2 drugs were determined. Whereas the whole blood concentration of methotrexate was determined, the plasma, and not the whole blood, concentration of probenecid was measured because of the limitations of the probenecid assay procedure. It was, of course, necessary to assume that the plasma concentration of probenecid was representative of the level of drug at the site where it was inhibiting the biliary secretion of methotrexate.

Using a least squares fitting program, the data were fit to Eq. 16, and a good fit was obtained ($r = 0.948$), Fig. 23. Each data point, with the exception of the point on the reciprocal clearance intercept, was from a different rat. The point on the intercept was the average value of four different studies, and therefore, for fitting the data, this point was appropriately weighted. The value of K_i obtained from this plot was 22.70 micrograms/ml.

The renal excretion data were not as readily evaluated as the biliary excretion data. One difficulty encountered was that urine samples, after attempts to dry them, contained an oily substance which would not oxidize completely. During the combustion step of the sample preparation procedure, this oily substance would vaporize and flow into the scintillation vial with the tritiated water. This resulted in extreme quenching of the

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FIGURE 22. Theoretical plot of reciprocal clearance as a function of the inhibitor concentration. The value of K_i can be obtained by dividing the value of the intercept of the reciprocal clearance axis by the slope.

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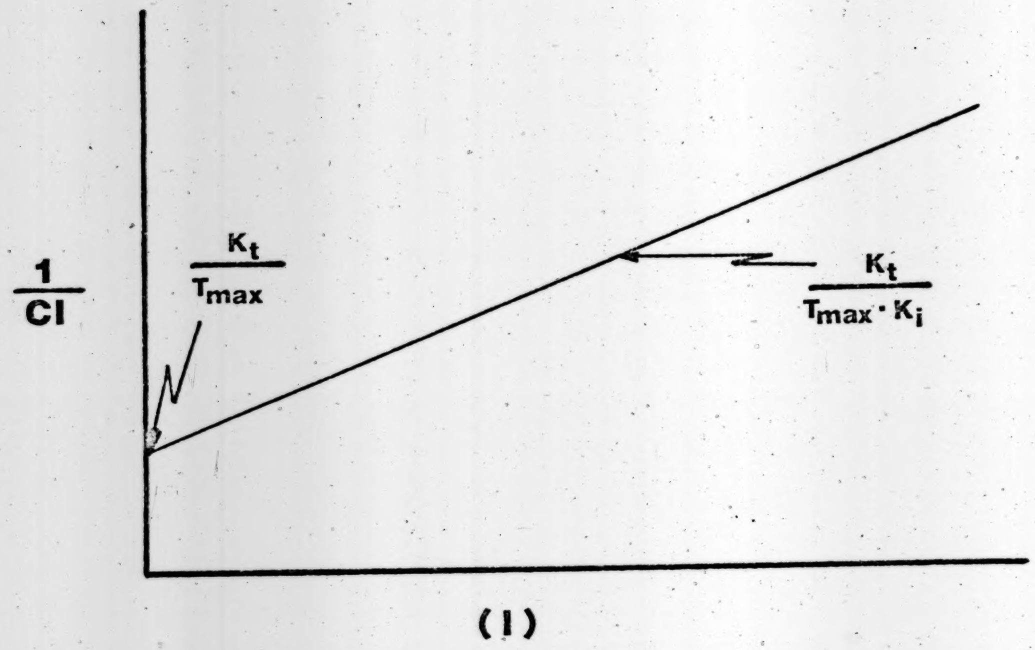

FIGURE 23. Plot of the reciprocal of biliary clearance of methotrexate as a function of probenecid plasma concentration. The value of K_i determined from this plot was 22.70 micrograms/ml. ($r = 0.948$). The data are given in Appendix IV.

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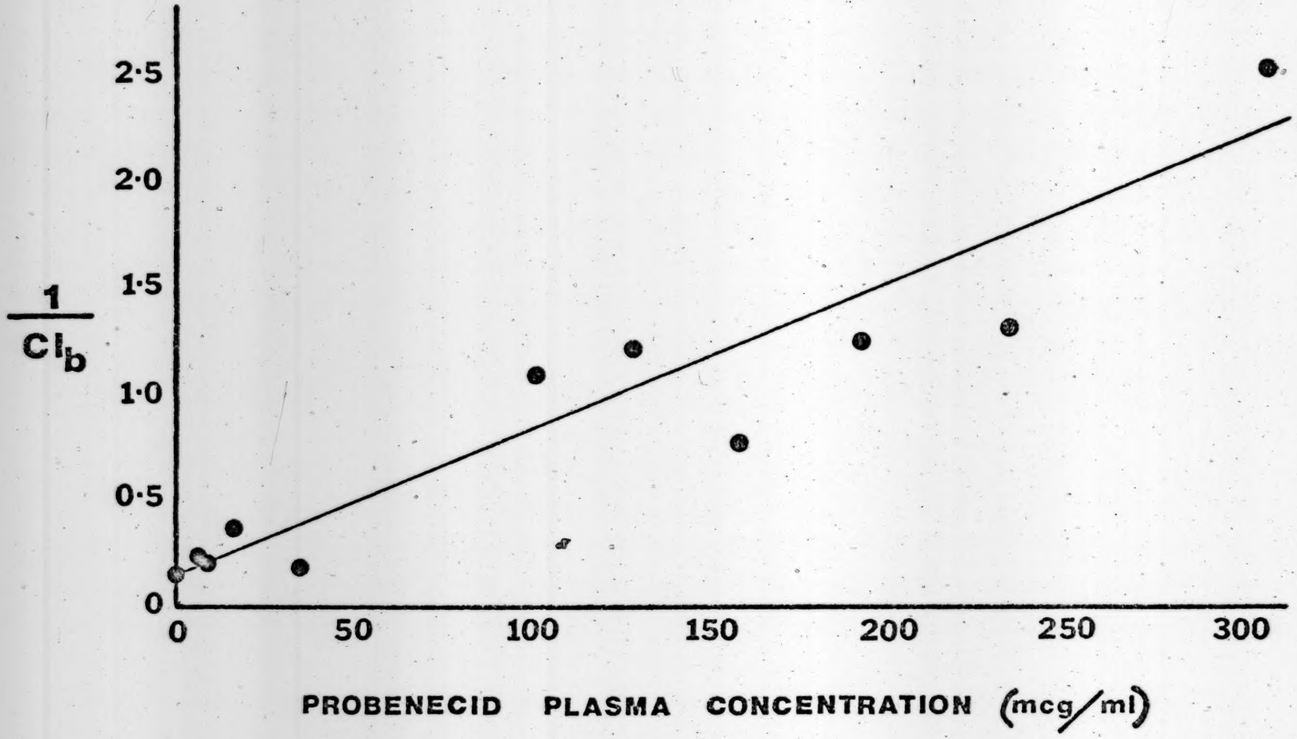
$\frac{1}{Cl_b}$
2·5
2·0
1·5
1·0
0·5
0
0
50
100
150
200
250
300
PROBENECID PLASMA CONCENTRATION (mcg/ml)

samples. In a few studies, the urine samples could not be analyzed with any degree of accuracy, even after attempts to dilute the sample in an effort to minimize the quenching.

In attempting to analyze the urine data, the contribution of filtration of methotrexate at the glomerulus and active secretion of the drug in the tubules were considered separately. A value of 6.1 ml/min./kg (63) was used as an estimate of the glomerular filtration rate in rats. Since methotrexate is approximately 50% bound to proteins in the plasma (64), the glomerular filtration rate was multiplied by 0.5 to get a value for renal clearance by filtration, and these values were then subtracted from the observed values of total renal clearance. As can be seen in Table X, the predicted values of renal clearance by filtration in 2 of the studies exceeded the observed values of total renal clearance. These data were, unfortunately, too inconsistent to be analyzed kinetically, and thus it was not possible to elucidate the parameters of the transport system involved.

The data from the previously discussed Clearance Study, suggest that probenecid inhibits the renal excretion of methotrexate and therefore leads to a decrease in renal clearance. The data obtained from measuring the urinary clearance of methotrexate at various plasma levels of probenecid also show that probenecid inhibits the renal elimination of methotrexate. These data are given in Table VI-3, in Appendix VI. Though the values of renal clearance appear to decrease as the concentration of probenecid is increased, the scatter of the data prevent any kinetic treatment from being performed.

Since the total body clearance of methotrexate was the important consideration, the inability to characterize the renal excretion mechanism was not considered to pose a serious problem. Instead of trying to treat

TABLE X

METHOTREXATE RENAL CLEARANCE DATA

Study	Cl_u^a	Cl_f^b	$Cl_f \cdot 0.5$	$Cl_u - Cl_f \cdot 0.5$
A	3.05	2.96	1.48	1.57
B	1.68	2.09	1.05	0.63
C	1.11	2.49	1.25	-0.14
D	0.12	2.64	1.32	-1.20
E	6.67	2.56	1.28	5.39
F	3.21	2.65	1.33	1.88

[a] Observed total urinary clearance. Units ml/min.

[b] Calculated renal clearance by filtration. Calculated as body weight multiplied by 6.1 ml/min./Kg. Units ml/min.

the urinary clearance data separately, the urinary and biliary clearances were looked at collectively, and it was assumed that their sum equaled total body clearance. A plot of the sum of urinary and biliary clearance as a function of the plasma concentration of probenecid is shown in Fig. 24. It can be seen that the total body clearance of methotrexate decreases as the plasma concentration of probenecid increases. As established previously, this decrease is primarily due to inhibition of the biliary secretion of methotrexate. From this plot, an estimation of the total body clearance of methotrexate can be made, if the plasma concentration of probenecid is known.

Methotrexate Bolus - Probenecid Infusion Studies

In the previously discussed studies, it was suggested that probenecid inhibits both the renal and biliary secretion of methotrexate and that the inhibition of the biliary secretion was by far the more significant effect. It was also shown that when methotrexate and probenecid were administered concurrently, the total body clearance of methotrexate could be predicted from knowledge of the plasma concentration of probenecid. In order to utilize this interaction clinically, it would be necessary to reduce the dose of methotrexate administered. With a lessened capability to eliminate methotrexate from the blood, a normally accepted dose would lead to excessive tissue exposure and result in increased toxicity. The objective of this group of studies was, therefore, to determine what modification in the dose of methotrexate would be required, in order that the modified dose of methotrexate (methotrexate administered with probenecid), would be therapeutically equivalent to a normally accepted dose.

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FIGURE 24. Plot of the sum of urinary (Cl_u) and biliary (Cl_b) clearances as a function of the plasma concentration of probenecid. The actual data are given in Appendix VI. The value of $Cl_u + Cl_b$ corresponding to a probenecid plasma concentration of zero is the average of four values (Studies A thru D). All other data points are from individual studies.

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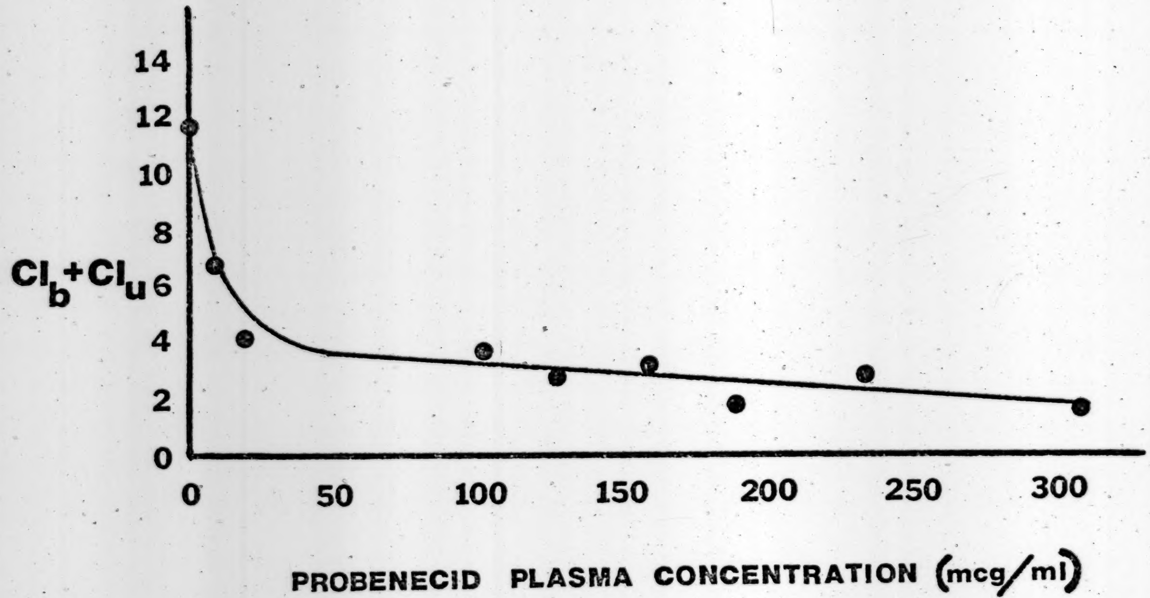
$Cl_b + Cl_u$
14
12
10
8
6
4
2
0
0
50
100
150
200
250
300
PROBENECID PLASMA CONCENTRATION (mcg/ml)

In order to propose a modified dose of methotrexate, it is necessary to have a common parameter or reference point for comparing the two. The ideal situation would be to compare the therapeutic and toxic responses to each dose; these, of course, are the ultimate considerations to be evaluated. A less direct means, however, was chosen because of the unavailability of leukemic rats, as well as the difficulty in evaluating drug induced lesions in the intestinal mucosa. The reference parameter which was selected for comparing a modified and a normal dose was the area under the blood concentration - time curve.

The area under the blood concentration-time curve is used generally for determining the physiological availability of drugs (65). This area is a function of both the dose administered and the total body clearance of the drug. This parameter is therefore well suited for the purposes intended here, as the objective is to evaluate a dose of methotrexate which has been modified because of a change in total body clearance. The relationship between the area under the blood concentration-time curve, the dose, and the total body clearance was briefly discussed in the section on the Methotrexate Bolus-Effect of Probenecid Studies. A rearrangement of equation one gives,

$$\text{Dose} = \text{Total body clearance} \times \text{Area} \qquad \text{(Eq. 17)}$$

A value for the total body clearance of methotrexate, as a function of the plasma concentration of probenecid can be determined from Fig. 25 if it is assumed that at a given plasma concentration of probenecid the clearance of methotrexate is constant. This necessitates giving doses of methotrexate which do not produce blood concentrations sufficient to approach saturation of the elimination processes. This relationship, therefore, is not valid at very high doses of methotrexate. Thus, for a given plasma concentration of probenecid, a dose of methotrexate can be calculated from equation 17 to give any desired area under the blood concentration-time curve.

In the previously discussed Methotrexate Bolus Studies, a series of five rats were injected intravenously with 25 mg/kg of methotrexate: the area under the blood concentration-time curve which was calculated from the blood level data obtained from these rats was 12.4 microgram-hr/ml. The area was calculated by fitting a triexponential equation using a weighted nonlinear least squares fitting program*, and then integrating the resulting equation.

The value for the area calculated by this procedure is approximately twice that calculated previously for the interval of from 15 minutes to 8 hours using the trapezoidal rule. The large difference between these two values is due primarily to the contribution to the total area of the first 15 minutes. Because of the extremely rapid decline in the blood concentration of methotrexate following an intravenous dose, a large percent of the total area is accounted for during the first quarter of an hour. Estimating the area under the blood concentration-time curve during this period of rapid decline in the blood concentration of methotrexate is subject to a great deal of error. Though a good fit of the data was obtained, there is no way of knowing how reliable the equation estimates the blood levels during the first 15 minutes. Because it was the most simple equation to give an acceptable fit, a triexponential equation was used to characterize the data. An equivalent fit, however, could be obtained using an equation with four or more exponential terms, while the

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* BMDX85T program - prepared at U.C.L.A. Science Computing Facility (66).

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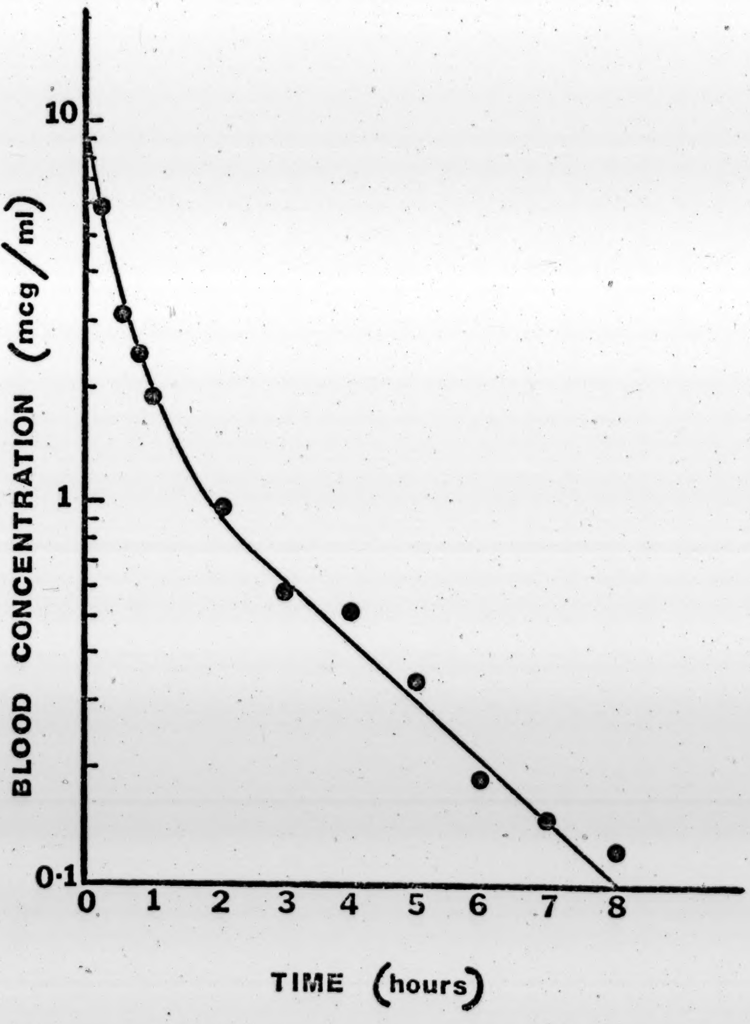
estimate of the blood levels during the first few minutes would be quite different. It is important to note that the total calculated area is extremely sensitive to the accuracy with which the blood concentration is estimated during the first 15 minutes following administration, and this is a potential source of significant error. Each data point was weighted by the reciprocal of the square of the concentration. Using the value for the normal total body clearance of methotrexate (in the absence of probenecid), an area of 15.5 microgram-hr/ml was calculated for a dose of 25 mg/kg from equation 17. The actually observed area is approximately 20% below this theoretical value.

The area under the blood concentration-time curve which was predicted for the 25 mg/kg dose was used as the reference point for calculating a modified dose of methotrexate. A probenecid plasma concentration of 230 microgram/ml, which corresponds to a total body clearance of methotrexate of 2.5 ml/min. was used. From the estimate of total body clearance, 2.5 ml/min., and an area of 15.5 microgram-hr/ml, a modified methotrexate dose of 5.3 mg/kg was calculated. The area under the curve resulting from this dose, when administered to a rat who has a probenecid plasma concentration of 230 mcg/ml, should be equivalent to the area under the blood concentration-time curve resulting from a 25 mg/kg dose administered in the absence of probenecid.

In order to evaluate the predictability of this system, a series of rats were infused with probenecid at rates which were anticipated to produce steady state plasma concentrations in the range of 230 mcg/ml, and then injected intravenously with 5.3 mg/kg of methotrexate. A blood concentration-time curve from one of these studies is shown in Fig. 25. It is of interest to note the difference between the shape of this curve and

FIGURE 25. Bolus of Methotrexate Administered To a Rat with a Constant Infusion of Probenecid. A rat was infused to a steady state probenecid plasma level of 194 mcg/ml and then administered 5.3 mg/kg of methotrexate as an intravenous bolus.

STUDY #3



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the blood concentration-time curve obtained when methotrexate is administered in the absence of probenecid (Fig. 11). The early rapid disappearance of drug from the blood which is observed in Fig. 11, is absent when probenecid is administered. In the absence of probenecid, the majority of the area occurs during the initial one or two hours after administration. With probenecid, the major contribution to area is the log-linear phase occuring after the initial distribution phase. The rapid disappearance of methotrexate following administration to nonprobenecid treated rats has been attributed to biliary secretion (67), and its diminished importance in the blood level picture of probenecid treated rats is not unexpected.

The data from the five probenecid treated rats is shown in Table XII. As mentioned previously, the dose of methotrexate was calculated from an estimated probenecid plasma concentration of 230 microgram/ml, and an intended area under the blood concentration-time curve (AUC) of 15.5 mcg-hr/ml. The values of predicted total body clearance (Cl_{tb}) were determined as a function of the measured probenecid plasma concentration using Fig. 25. Using these predicted values of total body clearance and the dose administered, an estimate of the area under the blood concentration-time curve was calculated from Eq. 17. The actual area under the curve was determined by fitting the blood concentration data to a biexponential equation, using the same fitting program as utilized for fitting the data from the nonprobenecid treated rats. The resulting equation was then integrated to give the area. The biliary clearance of methotrexate was determined as a function of the probenecid plasma concentration, and is expressed as a percent of normal. The blood concentration data for methotrexate, and the plasma concentration data for probenecid are presented

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TABLE XII

COMPARISON OF MODIFIED AND NORMAL DOSING OF METHOTREXATE USING AREA UNDER THE CURVE AS A REFERENCE

Study Number	Dose Administered[a] (mg/Kg)	Probenecid Plasma Level (mcg/ml)	Estimated[b] (Cl_{TB}) (ml/min)	Predicted[c] AUC (mcg-hr/ml)	Actual[d] AUC (mcg-hr/ml)	Biliary[e] Clearance (% of normal)
MTX[f] Alone	25	-	11.8	15.5	12.4	100
1	5.3	189	3.0	12.8	15.7	9.7
2	5.3	223	2.5	15.0	8.5	9.0
3	5.3	194	3.0	13.4	8.4	9.7
4	5.3	165	3.2	13.0	10.3	11.3
5	5.3	153	3.2	11.9	9.6	12.2

[a] The doses administered in studies 1 to 5 were calculated to give an area under the blood concentration-time curve of 15.5 mcg-hr/ml.

[b] These values were estimated from fig. 25, using the measured probenecid plasma levels.

[c] These values were calculated from Eq. 17 using the dose and the estimated Cl_{TB}.

[d] Calculated from Blood concentration data. These data are given in Appendix VII.

[e] Estimated from fig. 23.

[f] Average data from five rats used in the initial Methotrexate Bolus Studies.

in Appendix VII.

The probenecid plasma concentrations in each study were lower than the desired level of 230 mcg/ml, and the range of concentrations was from 153 to 223 mcg/ml. The probenecid plasma concentrations were all in the region where total body clearance of methotrexate is not very sensitive to changes in the probenecid plasma concentration. At lower plasma concentrations of probenecid, a variation in the probenecid level would lead to large changes in the total body clearance of methotrexate. The estimated total body clearance of methotrexate corresponding to the measured plasma levels of probenecid varied only slightly, and the range was from 2.5 to 3.3 ml/min.

The predicted area under the blood concentration-time curves were, in four of the five studies, greater than the actual area. Study No. 1 was the only study where the actual area was greater than the predicted area. The greatest difference between predicted and actual area was found in study number two, where the actual area was 43% less than the predicted area. The best agreement was found in study number five; the actual and predicted areas differed by only 19%. There are several possible sources of experimental error, or variation, which could account for the differences between the actual and predicted areas. These include: the estimation of the total body clearance of methotrexate from the probenecid plasma concentration data, the calculation of the area under the curve from the blood concentration data as discussed previously, as well as the possibility of assay errors.

In each study, the biliary clearance was estimated to be reduced to approximately 10% of normal.

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SUMMARY AND CONCLUSIONS

The results of this investigation can be summarized as follows:

(1) It was found that when probenecid is administered concurrently with methotrexate, the toxicity of methotrexate is greatly increased.

(2) It was then shown that probenecid inhibits both the renal and the biliary secretion of methotrexate, and that the inhibition of the biliary secretion process was by far the more significant effect of probenecid.

(3) In attempting to establish the identity of radiolabel in the plasma, it was shown that following an intravenous injection of tritiated methotrexate, the presence of a nonmethotrexate labeled component becomes significant with time.

(4) The kinetics of biliary transport of methotrexate were studied, and a value for the maximal transport rate and the transport constant were reported.

(5) The inhibition of the biliary transport of methotrexate by probenecid was investigated, and a value for the dissociation constant of the probenecid-transport carrier complex was determined.

(6) A graphical relationship between the total body clearance of methotrexate and the plasma concentration of probenecid was determined. The effect of probenecid on both the biliary and the renal excretion of methotrexate were taken into account.

(7) The final studies involved the evaluation of attempts to modify the dose of methotrexate. A modified dose was proposed, based on the degree of reduction of methotrexate's total body clearance by probenecid. The

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area under the blood concentration-time curve which was determined for a series of five rats was compared with a value predicted from the dose administered and an estimation of total body clearance.

What has been shown is that it is feasible to alter the physiological disposition of methotrexate by concurrently administering probenecid, and that the interaction between these two drugs can be quantified. The criteria used for evaluating this interaction took into account only pharmacokinetic considerations, and it is important to stress that studies conducted here do not justify any conclusions as to the therapeutic advantage of the proposed modified dosing procedure over the currently accepted methods for administering methotrexate. In evaluating this interaction between methotrexate and probenecid, it will be necessary to conduct a pharmacological study on leukemic rats and compare the therapeutic and toxic responses of the proposed modified dosing procedure with a currently accepted one.

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APPENDIX I

__Tables of Blood Concentration-Time Data Following a Single Intravenous Dose of Methotrexate.__ In studies one to five a single intravenous dose of methotrexate of 25 mg/kg was administered. In studies six to ten a 100 mg/kg dose of probenecid was administered intravenously 20 minutes before and 20 minutes after a bolus of 25 mg/kg of methotrexate.

TABLE I-1: Blood Concentration-Time Data For Experiment 1

Sample Time (hours)	Methotrexate Concentration (mcg/ml)
0.25	14.5
0.50	6.64
0.75	3.80
1	2.31
2	0.720
3	0.318
4	0.200
5	0.191
6	0.170
8	0.201

Rat weight _ 524 Gm

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TABLE I-2: Blood Concentration-Time Data For Experiment 2

Sample Time (hours)	Methotrexate Concentration (mcg/ml)
0.25	13.5
0.50	4.52
0.75	2.40
1	1.37
2	0.390
3	0.168
4	0.112
5	0.118
6	0.123
8	0.124

Rat weight - 472 Gm

TABLE I-3: Blood Concentration-Time Data For Experiment 3

Sample Time (hours)	Methotrexate Concentration (mcg/ml)
0.25	16.1
0.50	5.30
0.75	2.86
1	1.65
2	0.352
3	0.172
4	0.180
5	0.170
6	0.155
8	0.205

Rat weight - 482 Gm

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TABLE I-4: Blood Concentration-Time Data For Experiment 4

Sample Time (hours)	Methotrexate Concentration (mcg/ml)
0.25	13.0
0.50	4.37
0.75	2.24
1	1.44
2	0.405
3	0.179
4	0.219
5	0.154
6	0.094
8	0.115

Rat weight - 515 Gm

TABLE I-5: Blood Concentration-Time Data
For Experiment 5

Sample Time (hours)	Methotrexate Concentration (mcg/ml)
0.25	17.9
0.50	7.29
0.75	4.36
1	2.81
2	0.779
3	0.302
4	0.197
5	0.144
6	0.113
8	0.0926

Rat weight - 457 Gm

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TABLE I-6: Blood Concentration-Time Data For Experiment 6

Sample Time (hours)	Methotrexate Concentration (mcg/ml)
0.25	23.9
0.50	14.8
0.75	12.7
1	11.0
2	6.09
3	3.91
4	2.70
5	1.76
6	1.33
8	0.510

Rat weight - 522 Gm

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TABLE I-7: Blood Concentration-Time Data For Experiment 7

Sample Time (hours)	Methotrexate Concentration (mcg/ml)
0.25	31.5
0.50	10.8
0.75	8.60
1	8.13
2	5.41
3	2.95
4	1.58
5	0.737
6	0.460
8	0.228

Rat weight - 504 Gm

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TABLE I-8: Blood Concentration-Time Data For Experiment 8

Sample Time (hours)	Methotrexate Concentration (mcg/ml)
0.25	--
0.50	11.9
0.75	8.68
1	8.04
2	4.22
3	2.63
4	1.34
5	--
6	0.429
8	0.281

Rat weight - 492 Gm

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TABLE I-9: Blood Concentration-Time Data For Experiment 9

Sample Time (hours)	Methotrexate Concentration (mcg/ml)
0.25	34.9
0.50	--
0.75	--
1	7.42
2	3.75
3	2.19
4	0.833
5	0.333
6	0.138
8	0.224

Rat weight - 492 Gm

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TABLE I-10: Blood Concentration-Time Data
For Experiment 10

Sample Time (hours)	Methotrexate Concentration (mcg/ml)
0.25	29.0
0.50	16.0
0.75	13.9
1	11.5
2	12.7
3	4.47
4	2.95
5	1.18
6	1.12
8	0.538

Rat weight - 463 Gm

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APPENDIX II

Plasma Level Data For Studies On The Separation Of Methotrexate From Nonmethotrexate Components In Plasma. Table II-1 contains uncorrected methotrexate plasma concentration data. Table II-2 contains this plasma concentration data corrected for actual methotrexate. Table II-3 contains data on the percentages of label associated with methotrexate and nonmethotrexate components in plasma.

TABLE II-1: Plasma Concentration of Methotrexate - Not Corrected for Nonmethotrexate Components

Sample Time (hours)	Geometric Mean Plasma Concentration (mcg/ml)	Standard Error
0.25	24.2	1.17
0.50	12.2	1.02
1.0	5.80	1.50
2.0	1.78	1.10
4.0	0.385	1.23
6.0	0.247	1.27

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TABLE II-2: Plasma Concentration of Methotrexate - Corrected for Nonmethotrexate Components

Sample Time (hours)	Geometric Mean Plasma Concentration (mcg/ml)	Standard Error
0.25	24.2	1.17
0.50	12.2	1.02
1.0	5.51	1.02
2.0	1.55	1.09
4.0	0.277	1.21
6.0	0.165	1.28

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TABLE II-3: Percent of Label Associated With Methotrexate And Nonmethotrexate Components in Plasma

Study	% Methotrexate		% Nonmethotrexate	
	1 hr	2 hr	4 hr	6 hr
A	-	-	84/10	-
B	-	78/5	-	-
C	-	-	-	51/44
D	-	91/7	-	-
E	-	92/4	67/25	-
G	95/2	-	-	-
K	-	-	69/32	51/50
L	-	-	69/31	48/53
M	-	-	-	47/52
Average	95/2	87/5	72/25	49/50
Total	97	92	97	99

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APPENDIX III

Blood, Bile, and Urine Data From Methotrexate Clearance Study. Table III-1 contains the methotrexate blood concentration data from samples obtained at the midpoint of the urine and bile collection intervals. Table III-2 contains the biliary excretion data, and Table III-3, the urinary excretion data. Table III-4 gives the plasma concentrations of probenecid at specified sampling times.

TABLE III-1: Methotrexate Blood Level Data From Clearance Study

Sample Time (hours)	Blood Concentration (mcg/ml)
0.5	0.591
1.5	0.805
2.5	0.779
3.5	0.714
4.5	1.00
5.5	--
6.5	1.68
7.5	1.87
8.5	2.03
9.5	1.91
10.5	2.16
11.5	2.10
12.5	2.20
13.5	2.42
14.5	2.67

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TABLE III-2: Methotrexate Bile Excretion Data From Clearance Study

Sample Number*	Excretion Rate (mg/hr)	Biliary Clearance (ml/min)
1	0.40	11.3
2	0.29	6.0
3	0.25	5.4
4	0.26	6.1
5	0.26	4.4
6	0.25	--
7	0.14	1.4
8	0.14	1.3
9	0.14	1.2
10	0.13	1.3
11	0.12	1.0
12	0.13	1.0
13	0.12	0.9
14	0.11	0.8
15	0.12	0.8

*Sample interval was one hour.

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TABLE III-3: Methotrexate Urine Excretion Data From Clearance Study

Sample Number*	Excretion Rate (mg/hr)	Renal Clearance (ml/min)
1	0.27	7.6
2	0.20	4.1
3	0.21	4.6
4	0.21	5.0
5	0.21	3.4
6	0.38	--
7	0.34	3.4
8	0.37	3.3
9	0.37	3.1
10	0.34	3.0
11	0.32	2.5
12	0.36	2.9
13	0.34	2.6
14	0.34	2.3
15	0.36	2.3

*Sample interval was one hour.

TABLE III-4: Probenecid Plasma Concentrations For Clearance Study

Sample Time (hours)	Plasma Concentration (mcg/ml)
8.5	86.3
10.5	95.3
12.5	102.0
15.0	122.0

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APPENDIX IV

Biliary Secretion Data. Table IV-1 contains the data for each rat used in these studies. Tables IV-2, IV-3, and IV-4 contain the parameters plotted in the three linear transformations of the Michaelis-Menten equation. Table IV-2 gives values of 1/T and 1/(D), Table IV-3 gives values of (D)/T and (D), and Table IV-4 gives values of T and T/(D).

TABLE IV-1: Biliary Secretion Data

Study	Rat Weight (gm)	Methotrexate Blood Conc. (mcg/ml)	Biliary Excretion Rate (mg/hr)
A	486	7.98	2.04
B	343	16.20	3.66
C	408	57.40	8.05
D	432	60.50	7.40
E	420	9.05	3.48
G	434	264.00	11.40

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TABLE IV-2: Treatment of Data for Lineweaver-Burk Plot

Study	1/T[a]	1/(D)[b]
A	0.49	126.7
B	0.12	17.4
C	0.14	16.5
D	0.27	61.6
E	0.29	110.5
F	0.09	3.83

[a] Units of hr/mg.

[b] Units of ml/mg.

TABLE IV-3: Treatment of Data for the Woolf Plot

Study	(D)/T[a]	(D)[b]
A	3.91×10^{-3}	7.98
B	4.43×10^{-3}	16.20
C	7.13×10^{-3}	57.40
D	8.18×10^{-3}	60.50
E	2.60×10^{-3}	9.05
F	23.20×10^{-3}	264.00

[a] Units of hr/ml.

[b] Units of mcg/ml.

TABLE IV-4: Treatment of Data for Hofstee Plot

Study	T[a]	T/(D)[b]
A	2.04	256.0
B	3.66	226.0
C	8.05	140.0
D	7.40	122.0
E	3.48	385.0
F	11.40	43.2

[a] Units of mg/hr.

[b] Units of ml/hr.

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APPENDIX V

Transport Kinetic Studies--Data For Probenecid Inhibition of Methotrexate Biliary Transport. Table V-1 contains data for each rat in this study. The values of reciprocal biliary clearance, which are plotted in Fig. 23, are given in Table V-2 along with the corresponding plasma concentrations of probenecid.

TABLE V-1: Kinetic Transport Studies--The Effect of Probenecid

Study	Rat Weight (gm)	Methotrexate Blood Concentration (mcg/ml)	Methotrexate Bile Secretion Rate (mg/hr)	Probenecid Plasma Concentration (mcg/ml)
A	462	0.61	0.311	0.0
B	429	0.43	0.195	0.0
C	460	0.94	0.495	0.0
D	403	0.81	0.265	0.0
E	462	0.91	0.230	7.1
F	429	0.63	0.175	10.4
G	433	1.65	0.040	310.0
H	460	2.56	0.120	236.0
I	403	2.20	0.120	102.0
J	444	2.60	0.110	128.0
K	457	0.72	0.230	37.0
L	488	1.73	0.140	159.0
M	423	1.97	0.300	17.3
N	411	2.80	0.130	195.0

TABLE V-2: Values of Reciprocal Biliary Clearance Plotted in Fig. 23

Study	$1/Cl_b$*	Probenecid Plasma Concentration (mcg/ml)
A-D	0.14**	0.0
E	0.24	7.1
F	0.22	10.4
G	2.5	310.0
H	1.3	236.0
I	1.1	102.0
J	1.4	128.0
K	0.19	37.0
L	0.77	159.0
M	0.39	17.3
N	1.25	195.0

*Units of reciprocal clearance are min/ml.

**Average value.

APPENDIX VI

Renal Excretion Data For Methotrexate--The Effect of Probenecid. Table VI-1 contains the data used for calculating the values of clearance given in Table X. Table VI-2 contains the urinary excretion data obtained from studies on the effect of probenecid on the renal clearance of methotrexate. Table VI-3 contains the calculated values of urinary clearance.

TABLE VI-1: Excretion Rate and Blood Concentration Data Used in Calculation of Urinary Clearance

Study	Weight (gm)	Methotrexate Blood Conc. (mcg/ml)	Methotrexate Urinary Excretion Rate (mg/hr)
A	486	7.98	1.46
B	343	16.30	1.64
C	408	57.40	3.82
D	432	60.50	0.44
E	420	9.05	3.62
F	434	264.00	50.90

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TABLE VI-2: Renal Clearance of Methotrexate As a Function of the Plasma Concentration of Probenecid

Study	Weight (gm)	Methotrexate Blood Conc. (mcg/ml)	Urinary Excretion Rate (mg/hr)	Probenecid Plasma Conc. (mcg/ml)
A	462	0.61	0.17	0.0
B	429	0.43	0.09	0.0
C	460	0.94	0.26	0.0
D	403	0.81	0.21	0.0
F	429	0.63	0.09	10.4
G	433	1.65	0.12	310.0
H	460	2.56	0.37	236.0
I	403	2.20	0.34	102.0
J	444	2.60	0.30	128.0
K	457	0.72	0.08	159.0
L	488	1.73	0.15	17.3
M	423	1.97	0.11	195.0

TABLE VI-3: Effect of Probenecid on Methotrexate Renal Clearance

Study	Urinary Clearance (ml/min)	Probenecid Plasma Concentration (mcg/ml)
A	4.62	0.0
B	3.49	0.0
C	4.63	0.0
D	4.32	0.0
F	2.37	10.4
G	1.21	310.0
H	2.41	236.0
I	2.58	102.0
J	1.92	128.0
K	1.85	159.0
L	1.45	17.3
M	0.93	195.0

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APPENDIX VII

Data for Methotrexate Bolus - Probenecid Infusion Studies. Rats were infused with probenecid at a constant rate of 8.5 mg/hr, following a loading dose of 15 mg. Methotrexate was administered two hours after the start of the probenecid infusion as a single bolus of 5.3 mg/kg.

TABLE VII-1: Methotrexate Blood and Probenecid Plasma Concentration Data. Study #1

Sample Time (hours)	Methotrexate Blood Conc. (mcg/ml)	Probenecid Plasma Concentration (mcg/ml)
0.25	11.1	--
0.50	4.91	--
0.75	3.28	--
1.0	2.36	--
1.5	--	181.0
2.0	1.51	--
3.0	0.974	--
4.0	1.01	178.0
5.0	0.786	--
6.0	1.87	--
7.0	0.511	209.0
8.0	0.347	--

TABLE VII-2: Methotrexate Blood and Probenecid Plasma Concentration Data. Study #2

Sample Time (hours)	Methotrexate Blood Concentration (mcg/ml)	Probenecid Plasma Concentration (mcg/ml)
0.25	5.86	--
0.50	2.88	--
0.75	2.05	--
1.0	1.78	--
1.5	--	230
2.0	1.03	--
3.0	0.666	--
4.0	0.514	218
5.0	0.363	--
6.0	0.344	--
7.0	0.266	222
8.0	0.278	--

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TABLE VII-3: Methotrexate Blood and Probenecid Plasma Concentration Data. Study #3

Sample Time (hours)	Methotrexate Blood Concentration (mcg/ml)	Probenecid Plasma Concentration (mcg/ml)
0.25	5.97	--
0.50	3.07	--
0.75	2.50	--
1.0	1.88	--
1.5	--	195
2.0	0.962	--
3.0	0.575	--
4.0	0.506	196
5.0	0.333	--
6.0	0.185	--
7.0	0.150	190
8.0	0.123	--

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TABLE VII-4: Methotrexate Blood and Probenecid Plasma Concentration Data. Study #4

Sample Time (hours)	Methotrexate Blood Concentration (mcg/ml)	Probenecid Plasma Concentration (mcg/ml)
0.25	7.66	--
0.50	3.73	--
0.75	2.54	--
1.0	2.05	--
1.5	--	146
2.0	1.44	--
3.0	0.758	--
4.0	0.543	151
5.0	0.453	--
6.0	0.344	--
7.0	0.293	196
8.0	0.332	--

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TABLE VII-5: Methotrexate Blood and Probenecid Plasma Concentration Data. Study #5

Sample Time (hours)	Methotrexate Blood Concentration (mcg/ml)	Probenecid Plasma Concentration (mcg/ml)
0.25	7.14	--
0.50	3.28	--
0.75	2.79	--
1.0	2.17	--
1.5	--	159
2.0	0.907	--
3.0	0.801	--
4.0	0.504	138
5.0	0.402	--
6.0	0.259	--
7.0	0.211	163
8.0	0.187	--

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